Amino Acid Incorporation by Preparations from the Developing Rat Brain

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1. A study has been made of the ability of cerebral microsome-cell sap systems, taken from rats at various ages, to incorporate [^{14}C]valine. 2. The systems appear to follow Michaelis–Menten kinetics when cell sap is considered as substrate, microsomes as enzyme and total counts/mg. of microsomal protein as a measure of reaction velocity. 3. Reproducible 'affinity constant' values are obtained, and the system from 4-day-old rats has a higher $V_{max.}$ and affinity constant than the system from adult rats. 4. It is suggested the the amino acid-incorporating ability of different systems may be compared by this means.

In the brain the metabolism of protein appears similar to that in other organs. It also appears generally agreed that *in vivo* the rate of protein synthesis is higher in foetal or immature brain than in adult brain (Gaitonde & Richter, 1956; Lajtha, Furst, Gerstein & Waelsh, 1957; Lajtha, 1959, 1964). However, attempts to show this difference *in vitro* with the microsomal-transfer RNA amino acid-incorporating system have resulted in conflicting reports. Bondy & Perry (1963) and Murthy & Rappoport (1965), using mitochondria-free preparations, reported that the rate of amino acid incorporation was higher in newborn than in adult systems. However, Suzuki, Korey & Terry (1964) found that there was no great difference when grey matter was used, the only significantly higher rate being obtained with white-matter preparations.

According to Gelber, Campbell, Deibler & Sokoloff (1964) and Klee & Sokoloff (1964) differences in the rate of incorporation by young and adult systems could be observed only when mitochondria were added as a source of ATP. The differences were abolished by the presence of a system generating adequate amounts of ATP.

In general therefore these reports provide no evidence that the amino acid-incorporating system from young animals has any intrinsically greater synthetic ability. However, there seems to have been little attempt to establish a rational basis for comparison, and in particular to ensure that the young and adult systems were operating under optimum conditions.

The present paper describes an attempt to establish a basis for the comparison of the amino acid-incorporating abilities of different systems.

MATERIALS AND METHODS

Animals. Wistar albino rats were bred in this Institute and used 3–4 days after birth (wt. about 7g.), or as weanlings (about 50g.), or as young adults (about 200g.).

Chemicals. The disodium salts of ATP and creatine phosphate were purchased from the Sigma Chemical Co. DL-[1-^{14}C]Valine was obtained from The Radiochemical Centre, Amersham, Bucks., and used undiluted (33·9μc/μmole).

Preparation of homogenates and subcellular fractions. The rats were killed by decapitation, the brain was rapidly removed and the cerebral cortex dissected out. The cortices were pooled and homogenized in 0·25M-sucrose, 0·5mM-$MgCl_2$ (pH7·2) in a glass homogenizer with a Teflon pestle. Homogenization was effected by rapidly moving the pestle up and down nine times.

The homogenates were centrifuged as follows. (1) 15000g for 10min. The supernatant was removed, shaken and recentrifuged in the same way. (2) The final supernatant from (1) was centrifuged at 105000g for 90min. The pellet constituted the microsome–ribosome fraction, and the upper two-thirds of the supernatant the cell-sap fraction. Centrifugation was carried out in a Spinco model L ultracentrifuge (no. 40 head).

The microsome pellet was carefully rinsed several times with a buffered sucrose solution (0·25M-sucrose, 0·025M-KCl, 0·5mM-$MgCl_2$, 0·05M-tris, pH7·6) and finally resuspended in fresh buffer.

Incubation conditions. Our aim was to stimulate conditions *in vivo* as closely as possible. The resuspended microsomes were therefore incubated with whole cell sap with the minimum of additives. The complete mixture contained known amounts of microsomes and cell sap together with final concentrations of 10mM-$MgCl_2$, 20mM-KCl, 1mM-ATP, 20mM-creatine phosphate, 20mM-tris (pH7·6). Finally 0·5μc of DL-valine was added, the volume made up to 1ml., and the mixture incubated at 37° for 30min. The reaction

was stopped by the addition of ice-cold trichloroacetic acid (final concentration 5%) containing 0·05 M-DL-valine. In cases where varying amounts of cell sap were used, additional cell sap was added before the trichloroacetic acid to give a uniform protein concentration.

Purification and radioassay of protein. The trichloroacetic acid-precipitated protein was washed with 5% trichloroacetic acid containing 0·05 M-valine. The trichloroacetic acid washing was continued until no counts could be detected in the wash. RNA was removed by heating the precipitate with 5% trichloroacetic acid for 15 min. at 90°. Trichloroacetic acid was removed by washing three times with 95% ethanol. The residue was then extracted at room temperature twice with chloroform–methanol (2:1), once with ethanol–ether (1:1) and finally with ether and allowed to dry. The dry protein was dissolved in 0·25 ml. of formic acid (98%) at 70°. The solution was transferred to aluminium planchets, the formic acid evaporated by a warm air stream, and the radioactivity of each sample estimated by standard techniques with a Nuclear–Chicago continuous-gas-flow end-window counter (efficiency 30%).

Recovery and estimation of protein. After counting, the protein was eluted from the planchets with 98% formic acid, and the planchets were counted again to ensure complete recovery. The formic acid was allowed to evaporate, and the residual protein dissolved in 0·1 N-NaOH, and estimated in triplicate samples by the method of Lowry, Rosebrough, Farr & Randall (1951). Bovine serum albumin similarly dissolved in formic acid was used as a standard.

Estimation of RNA. For the determination of RNA/protein ratios in the microsome fraction the RNA was extracted and estimated by the method of Dingman & Sporn (1962).

Valine pool sizes. Cell-sap samples were taken from groups of six rats of the various age groups used, and precipitated with trichloroacetic acid. The valine in the resulting supernatants was estimated with a Technicon automatic amino acid analyser (Hamilton, 1963).

RESULTS

Table 1 shows that the RNA/protein ratio of microsome preparations from rat brain of different ages was sufficiently constant to allow the incorporation of amino acid to be given in terms of microsomal protein content rather than RNA content. The results are similar to those reported by Kirsch, Siekevitz & Palade (1960) for guinea-pig-liver microsomes.

Linearity of incorporation. A number of experiments were done to check the time-course of valine incorporation. As shown in Fig. 1, this appeared to be essentially linear for 30 min. and hence the incorporation observed over 0–30 min. has been taken as a measure of the reaction velocity.

Pool-size correction. The valine pool sizes in the cell sap taken from rats of different ages are given in Table 2 and show that the cerebral cortex of 4-day-old rats contains about twice as much as that of adults.

When constant amounts of microsomes taken from rats of different ages were incubated as described, with varying amounts of cell sap, the rate of incorporation of [^{14}C]valine reached a peak and then declined as shown in Fig. 2. When, however, the appropriate correction was made for dilution of the valine by the endogenous pool, the incorporation reached a plateau. In our system therefore it seemed unnecessary to follow the technique of Sephadex treatment of the cell sap claimed by Munro, Jackson & Korner (1964) to be necessary for the purpose of removing inhibitors present in cell sap. In all subsequent experiments therefore, the results are given after correction for endogenous pool dilution.

Table 1. *Protein and RNA content of rat cerebral cortex microsomes at various ages*

Results are given in mg./g. wet wt. ± S.E.M.

	4-Day-old	Weanling	Adult
Protein	11·1 ± 0·5	4·2 ± 0·3	3·1 ± 0·2
RNA	1·2 ± 0·1	0·48 ± 0·03	0·34 ± 0·02
RNA/protein	0·108	0·113	0·109

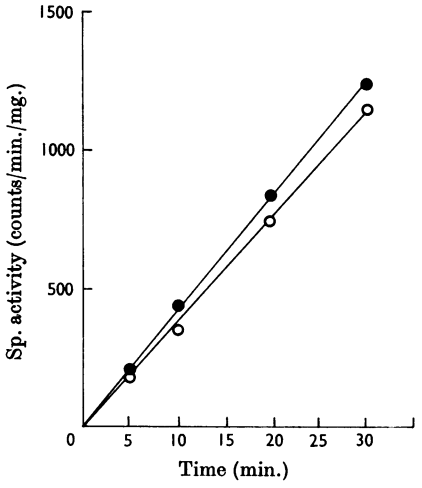
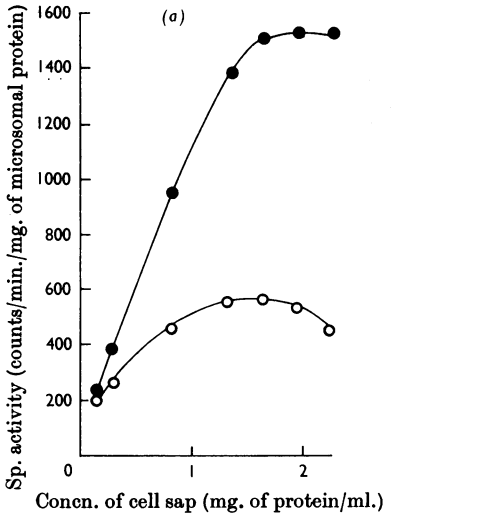


Fig. 1. Incorporation of [^{14}C]valine into protein by cerebral microsomes from 4-day-old and adult rats over a 30 min. period: ●, 4-day-old; ○, adult.

Table 2. *Valine pool sizes in rat cerebral cell sap at various ages*

Results are for pooled cell sap from six rats.

	Valine pool size (μmole/g. wet wt. of cerebral cortex)		
Expt.	4-Day-old	Weanling	Adult
1	0·123	0·084	0·058
2	0·135	0·072	0·058



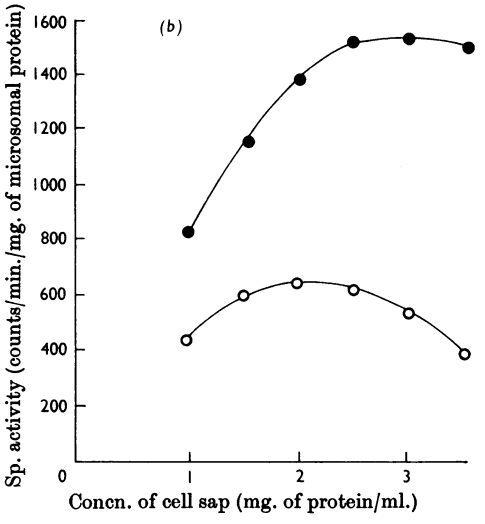


Fig. 2. Effect of increasing cell-sap concentrations on amino acid incorporation by cerebral microsomes. (*a*) System *in vitro* from 4-day-old rat. (*b*) System *in vitro* from adult rat. ○, Uncorrected and ●, corrected, for dilution of [^{14}C]valine by endogenous valine in cell sap.

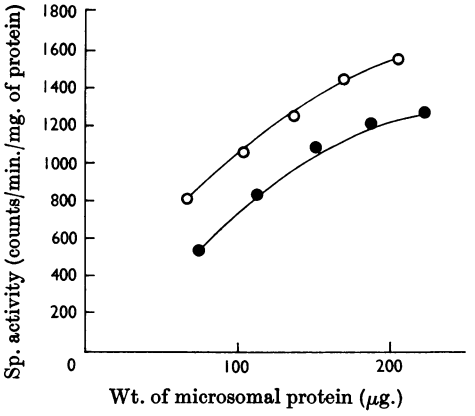


Fig. 3. Effect of maintaining a constant ratio of cell-sap protein to microsomal protein while the concentration of microsomal protein is raised. The ratio of cell sap/microsome protein was kept at 8:1. ●, Adult rat cerebral cortex; ○, 4-day-old rat cerebral cortex.

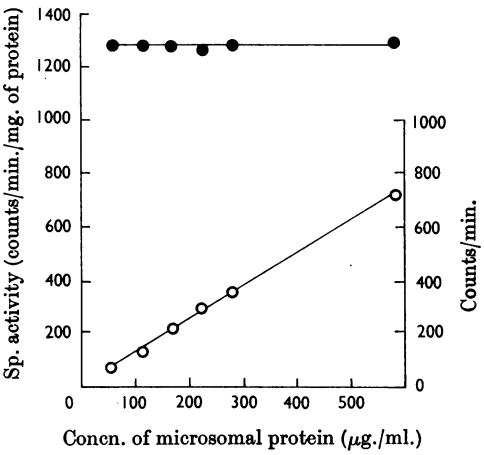


Fig. 4. Effect on [^{14}C]valine incorporation of varying the concentration of microsomal protein with constant cell-sap concentration (adult rat cerebral cortex). The concentration of cell-sap protein was 2·3 mg./ml. ●, Specific activity (counts/min./mg. of protein); ○, counts/min.

From the results in Fig. 2 there appeared to be no obvious difference between the incorporating ability of the systems prepared from rats of different ages.

Optimum ratios of microsomes/cell sap. Many investigators describe optimum conditions for incorporation in terms of a ratio of microsomes/cell sap. With the microsome/cell sap ratios at which the beginning of the incorporation plateau was reached (Fig. 2), the concentration of microsomes (and therefore of cell sap) was varied. The results (Fig. 3) showed that the specific activity of the protein in both 4-day-old and weanling rat systems increased with increasing concentrations of microsomal protein. Maintaining a constant ratio of microsomes/cell sap did not therefore give a constant incorporation at all concentrations of microsomes. In subsequent experiments varying amounts of microsomes were incubated with constant amounts of cell sap. The results (Fig. 4) showed that under these conditions the total counts/min. in the protein was proportional to the amount of microsomes present, but that the specific activity was constant.

The shape of the curves in Fig. 2 and the results

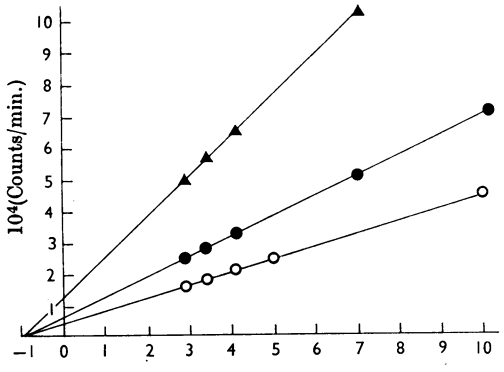


Fig. 5. Reciprocal plots of total counts/min. and cell-sap concentration. Values are given for three different concentrations of microsomal protein (adult rat cerebral cortex): ▲, 158 μg./ml.; ●, 316 μg./ml.; ○, 475 μg./ml.

given in Figs. 3 and 4 suggested that the relationship between microsomes and cell sap might be similar to an enzyme–substrate interaction following Michaelis–Menten kinetics. Consequently three different amounts of microsomes from an adult cerebral preparation were incubated with varying quantities of cell sap. The results were plotted as reciprocals of total counts/min. against cell sap concentration (Fig. 5). Straight-line plots were obtained, which, when produced, cut the total counts/min. ordinate at points whose reciprocals were proportional to the microsome concentrations. The lines also coincided on the abscissa to give an 'affinity constant' value. The results were repeated with preparations from 4-day-old rats, and were similar in pattern to those in Fig. 5, except that the plots coincided on the abscissa at a point closer to the origin. This difference between young and adult systems was confirmed by further experiments in which microsomes and cell sap from both systems were mixed and matched. The results (Fig. 6) showed that the young microsomes–young cell sap combination had a higher 'affinity constant' than the other possible combinations. Adult microsomes with young cell sap behaved similarly to the adult system in Fig. 5. Additional experiments (details as in Figs. 5 and 6) with microsomes from 4-day-old and adult animals combined with cell sap from young animals gave the following results (six results/group ± S.D.): the 'affinity' constant for adults was 0·104 ± 0·012 and for 4-day-olds 0·054 ± 0·0043, the difference being highly significant.

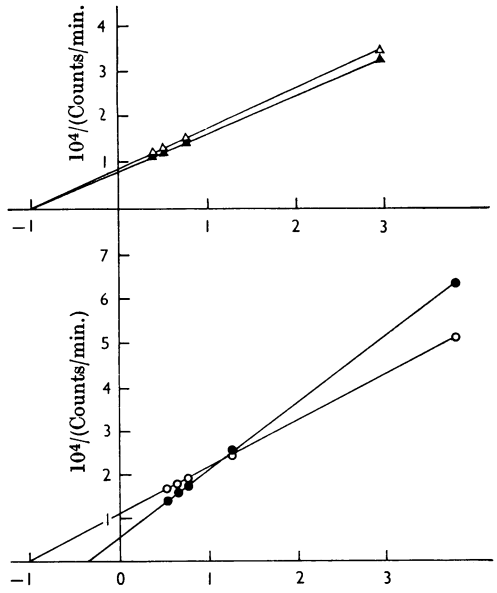


Fig. 6. Mix-and-match experiment with microsomes and cell sap from 4-day-old and adult rat cerebral cortex. The results were plotted as in Fig. 5. The concentration of microsomal protein was kept constant at 0·5 mg./ml. △, Adult microsomes and adult cell sap; ▲, 4-day-old microsomes and adult cell sap; ○, adult microsomes and 4-day-old cell sap; ●, 4-day-old microsomes and 4-day-old cell sap.

DISCUSSION

At first sight the cell-free systems prepared from the brain of rats of varying ages appeared to be similar in their ability to incorporate [^{14}C]valine. Fig. 3 shows that the maintenance of a constant ratio of cell sap to microsomes did not give a constant value for counts/min./mg. of microsomal protein and consequently that different systems cannot be compared simply by using similar ratios. Further the counts/min./mg. of microsomal protein were dependent on the amount of cell sap present (Fig. 4), and when the cell sap concentration was held constant the total counts/min. increased proportionally to the concentration of microsomes. However, the specific activity of the microsomal protein remained constant. This is the general pattern which would be expected of a classical enzyme–substrate system if the cell sap is acting as substrate and the microsomes as enzyme. From the shape of the curves in Fig. 2 it also appeared that the system might follow Michaelis–Menten kinetics according to the relationship:

$$\text{Microsomes (E)} + \text{cell sap (S)} \underset{K_1}{\overset{K_2}{\rightleftharpoons}} \text{microsome–cell sap complex (ES)} \rightarrow \text{peptide chain (products)}$$

the products being measurable in terms of counts/min. incorporated. When 1/(cell sap concentration) was plotted against 1/(counts/min.) straight lines were obtained with both young and adult systems, which, when extrapolated, cut the $1/V$ axis at points giving $V_{max.}$ values proportional to the microsome concentrations. The lines also converged at single points on the abscissae giving apparent 'affinity constants' which differed for the two systems. Haselkorn & Fried (1964) made a similar approach in their studies on the attachment of polyuridylic acid and RNA obtained from tobacco mosaic virus to free ribosomes.

The suggestion of a higher affinity of microsomes from young animals for cell sap from the same source was confirmed by the results in Fig. 6 and in the Results section. The affinity also seems influenced by a cell-sap factor since microsomes from young animals behaved like those from adults when combined with adult cell sap.

The results suggest therefore that microsomes from young animals have an inherently greater capacity for amino acid incorporation than those from adults.

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