Metabolically Inert Proteins of the Central and Peripheral Nervous System, Muscle and Tendon

By A. N. DAVISON*

Department of Biochemistry, Guy's Hospital Medical School, London, S.E. 1

(Received 29 June 1960)

It has recently been suggested that brain constituents, in common with the constituents of other tissues, may be roughly divided into at least two main groups according to their predominant rates of turnover (Davison & Dobbing, 1959). These two groups are represented by a metabolically active compartment and a more permanent compartment associated with structural elements such as the myelin sheath. Evidence in support of this concept comes from studies of lipid metabolism *in vivo* in the central nervous system. It was shown by labelling with ^{14}C and ^{32}P that part of the cerebral cholesterol and phosphatide undergoes turnover whereas the remainder is metabolically stable (Davison, Morgan, Wajda & Payling Wright, 1959; Davison & Dobbing, 1959, 1960*a, b*). Lipid undergoing turnover was found mainly in the grey matter, whereas the metabolically inert lipid was located predominantly in the white matter (Davison & Dobbing, 1960*b*; Davison, Dobbing, Morgan & Payling Wright, 1959). Furthermore, radioactivity was found to persist in those lipids which are characteristic of the myelin sheath.

It is known that the myelin sheath contained protein and lipid arranged as laminae around the axon (Finean & Robertson, 1958), and since the lipids of the myelin sheath appear to be inert it seemed possible that these myelin proteins may also be metabolically stable. Since at least a part of the myelin protein is of proteolipid nature (Folch & Lees, 1951; Koenig, 1959), it seemed of interest to investigate the metabolism of neural proteolipids and other proteins by using $[1-^{14}\text{C}]$ -glycine as a suitable precursor for proteolipid protein (Folch & Lees, 1951; Uzman, 1958; Uzman & Rosen, 1958) and for other constituents.

In the present study radioactive glycine was injected into developing rats and became incorporated into both non-structural and structural elements, such as the myelin sheath and tendon. The fate of the labelled proteins was followed at intervals up to 250 days after the initial injection, by which time the rats were fully grown. By this means it was possible to distinguish between metabolically active and stable groups of protein.

* Member of the external scientific staff of the Medical Research Council.

Adult rats were also given a single injection of $[1-^{14}\text{C}]$ -glycine, and the turnover of labelled protein was followed for 197 days. In conformity with the present hypothesis it was found that in the adult all the labelled proteins were metabolically active.

MATERIALS AND METHODS

Animals. Nine 11-day-old rats (25–30 g.) from two litters were each given 0.25 ml. of 0.9% (w/v) sodium chloride solution containing $5\mu\text{C}$ of $[1-^{14}\text{C}]$ -glycine by intraperitoneal injection. Six female adult rats (320–360 g.) were also given an intraperitoneal injection of $16.6\mu\text{C}$ of $[1-^{14}\text{C}]$ -glycine in 0.5 ml. of 0.9% (w/v) sodium chloride solution. Animals were killed at intervals up to 250 days after the injection. Blood was withdrawn from the heart. Brain and spinal cord, sciatic nerve, tail tendon and the quadriceps femoris muscle were removed, washed in saline, dried with filter paper and weighed.

Extraction of nervous tissue

Nervous tissue was homogenized in 20 vol. of chloroform-methanol (2:1, v/v) and the extract filtered. The extract was then washed with 0.2 vol. of water as described by Folch, Lees & Sloane-Stanley (1957). The two phases were allowed to separate, a portion of the upper phase was dried, and radioactivity was determined either on a planchet, as for blood (see below), or after dispersing in xylene plus phosphor, by scintillation counting. Traces of radioactivity remained detectable in the upper phase up to 9 days after injection, so that in rats killed within this time the lower phase, which contained the total lipid and proteolipid, was washed a second time with an equal volume of synthetic upper phase (Folch *et al.* 1957).

Treatment of muscle, tendon and kidney

Muscle protein. Approx. 1 g. of muscle was washed in 0.9% sodium chloride and was dispersed in 20 ml. of 5% (w/v) trichloroacetic acid with the aid of a tissue blender. The precipitated protein was centrifuged down and the procedure repeated until the supernatant was free of radioactivity. The precipitate was then washed with 10 ml. of ethanol and twice extracted with 20 ml. of ethanol-ether (3:2, v/v) at room temperature for 30 min., washed with 10 ml. of diethyl ether and dried *in vacuo*. Some of the dried precipitated protein was macerated with 0.1 N-NaOH for 24 hr., and the alkali-insoluble residue was washed with water and ethanol and then dried *in vacuo* (Lowry, Gilligan & Katersky, 1941). Hydroxyproline estimations indicated that this insoluble residue contained most of the muscle collagen.

Tendon. Tail tendon was washed in 0.9% sodium chloride and cut finely with scissors. The tendon was then treated with 5% (w/v) trichloroacetic acid, washed and dried as described for muscle protein.

Kidney. One kidney was removed, washed free of blood and dried on a filter paper. The kidney was dispersed in 20 vol. of chloroform-methanol (2:1, v/v) and the extract filtered. The residue was washed with 10 ml. of ethanol and then suspended in 25 ml. of water. After standing for 30 min. at room temperature, the suspension was filtered and the residue washed with 10 ml. of ethanol and dried *in vacuo*.

Measurement of radioactivity

Estimation of radioactivity in blood. Blood (0.1 ml.) and plasma (0.1 ml.) were allowed to dry on planchets (1.5 cm. diam.) and radioactivity was determined in a thin-window (mica) Geiger-Müller counter. To follow the persistence of the radioactive glycine in the blood, 0.5 ml. of plasma was also treated with 0.1 ml. of 50% (w/v) trichloroacetic acid and, after centrifuging, 0.1 ml. of protein-free supernatant was dried on a planchet and radioactivity determined.

Scintillation counting. Radioactivity was measured by liquid scintillation counting with the Ekco N612 counter operating at room temperature (Davison, Morgan, Wajda & Payling Wright, 1959; Stitch, 1959). Xylene (May and Baker Ltd., reagent grade) was employed as solvent with 2:5-diphenyloxazole (0.3%, w/v) as scintillator solute. Poly¹⁴Cmethyl methacrylate standards of known radioactivity and background samples were counted at intervals throughout the day. There was no indication of instrument drift. Most samples were counted for a total of 10–15 min., but with a few preparations, the radioactivity of which was less than twice background activity (30 counts/min.), counting a sample and background was continued for 60 min.

Determination of radioactivity in total lipid plus proteolipid fraction. The background count of a scintillation-counting cell containing 20 ml. of xylene plus phosphor was determined. The xylene mixture was then poured into a

beaker and a suitable volume of the initial chloroform-methanol extract [extract (D), Fig. 1] transferred to the counting cell. The solvent (chloroform-methanol) was removed by passing a stream of nitrogen into the pot. A few drops of methanol were added towards the end of the evaporation to prevent the formation of two phases. Final drying was effected on a water bath at 60°.

After cooling, the 20 ml. of xylene plus phosphor that had been used for the background count was returned to the pot to take up the dried lower-phase deposit, and radioactivity was determined. Traces of insoluble protein in some samples did not cause any loss of radioactivity.

Determination of radioactivity of protein-free lipid. The remaining chloroform-methanol extract was then mixed with an equal volume of synthetic upper phase containing citrate (G. R. Webster & J. Folch, in preparation). After 30 min. insoluble strands of protein separated at the interface between the two phases (stage 4, Fig. 1). This protein, derived from the proteolipid, was removed. No radioactivity was detected in the citrate-containing upper phase after decomposition of proteolipid. A suitable volume of the protein-free lower phase (E) was dried in a flask, and the lipids were dissolved in 20 ml. of xylene plus phosphor. The lipid solution was then transferred to a scintillation counting cell in which background count had already been determined. Radioactivity was measured by scintillation counting (Davison, Morgan, Wajda & Payling Wright, 1959). It was found unnecessary at this stage to wash the lower phase again with synthetic upper phase.

Determination of radioactivity of protein from proteolipid of central nervous system. The material (F), separated from the lower phase after treatment with upper phase containing citrate, was washed with chloroform-methanol (2:1, v/v) and the washings were added to the lower phase. About 2 ml. of ethanol was added to the protein residue, which was then washed with 10 ml. of water. The residue was finally treated with about 5 ml. of acetone and dried in a vacuum desiccator. Although the dry denatured protein residue was ground in a mortar it proved very difficult to produce a suitably fine powder. Particles were mixed with 10 ml. of scintillating gel NE 210 [Nuclear Enterprises

Fig. 1. Treatment of tissue of central nervous system. This figure illustrates the procedures adopted for the separation of nervous tissue. In this experiment wet weights of adult rat brain and spinal cord are given, all other figures are dry weights.

(G.B.) Ltd., Sighthill, Edinburgh 11] in a sample pot and while stirring, the mixture was allowed to gel by warming on a water bath. Radioactivity was determined by scintillation counting.

Radioactivity of brain and spinal cord residues after chloroform-methanol extraction. Residues (A) (Fig. 1), after extraction of lipid, were exhaustively washed and dried as described by LeBaron & Folch (1956). Radioactivity in this dried powder (B) (Fig. 1) was determined in a thin-window Geiger-Müller counter by an infinite-thickness technique and also by counting the radioactivity of a suspension in gel NE 210 with the scintillation counter. The dried powder (50 mg.) was treated with trypsin solution (stage 3, Fig. 1) for a fortnight (LeBaron & Folch, 1956) and then washed with dilute acid, water and acetone before drying. The radioactivity of a suspension of the dried trypsin-resistant protein fraction was determined in gel NE 210.

Validity of the scintillation-counting methods

Since a number of different procedures have been applied to the lipid extract in order to decompose proteolipid, it was necessary to ascertain whether or not such treatment interfered in the determination of radioactivity by scintillation counting. For this purpose an internal standard of ^{14}C -labelled glyceride was added to 20 ml. of xylene plus phosphor containing (1) lipid + proteolipid, (2) washed lipid extract, (3) citrate-treated lipid extract. There was no evidence of any quenching in any of the three preparations.

Protein residues. These were counted as a suspension in a scintillation gel. The validity of this method was investigated in several ways.

Fig. 2. Radioactivity of ^{14}C -labelled protein suspended in scintillation gel. Different amounts of radioactive protein residue from the central nervous system of an adult rat injected with $[1-^{14}\text{C}]\text{glycine}$ 15 days earlier have been suspended in scintillation gel. In the first experiment (●) unlabelled protein powder was added to each sample pot to make the total protein content up to 50 mg. and radioactivity was determined by scintillation counting. In the second experiment (×) different amounts of radioactive protein (3.4–50 mg.) were weighed into each sample pot and radioactivity was measured without dilution.

(i) Internal quenching. There was no loss of radioactivity when an internal ^{14}C standard was added to a suspension of 50 mg. of protein powder.

(ii) Particle size. Radioactive protein was powdered in a mortar and some of the powder was passed through a fine sieve (200 mesh). There was only a slight gain (3.5%) of radioactivity in 50 mg. of fine compared to the unsieved powder.

(iii) Sample size. Different amounts up to 50 mg. of radioactive protein powder diluted with non-radioactive protein to give 50 mg. of protein gave a proportionate increase in radioactivity (Fig. 2). Different amounts up to 50 mg. of undiluted radioactive protein powder gave similar increases in radioactivity.

(iv) Comparison with Geiger-Müller counting. Finally the reliability of the technique was checked by comparing the proportion of radioactivity found with the Geiger-Müller counter with that with the scintillation counter (Table 2). The rates of loss of radioactivity are comparable but scintillation counting, operating with a much greater efficiency, gives more consistent results.

Analytical methods

Protein. This was determined with bovine albumin as a standard by the biuret method (Robinson & Hogden, 1940) as modified by Aldridge (1957). Amino acids from protein hydrolysates were separated by two-dimensional chromatography on buffered paper with butanol-acetic acid-water (4:1:5, by vol.) and *m*-cresol-phenol (1:1, v/v) as solvents (Levy & Chung, 1953).

Nitrogen and phosphorus. These were determined as described by Davison & Wajda (1959).

Hydroxyproline. Hydroxyproline was assayed by the method of Neuman & Logan (1950) on protein residues hydrolysed in 1 ml. of 6*N*-HCl in a sealed tube for 6 hr. at 110°. Collagen contains 14.5% of hydroxyproline (Neuberger, 1955), and since this amino acid is not present in other groups of protein it was possible to calculate the approximate collagen content of a specimen.

RESULTS

In this work the incorporation and persistence of radioactivity in the proteins of the central and peripheral nervous system, muscle and tendon have been followed for long periods after intraperitoneal injection of $[1-^{14}\text{C}]\text{glycine}$ into neonatal and mature groups of rats. In an earlier experiment in which ^{32}P was used in metabolic studies of cerebral phospholipids (Davison & Dobbing, 1960*a*) ^{32}P -labelled precursors remained in the blood for long periods. Since such persistence of radioactive precursor makes interpretation of long-term metabolic studies difficult, levels of radioactivity in whole blood and plasma were followed in all animals injected with labelled glycine. To exclude the possibility that this radioactivity is due to persistence of non-protein ^{14}C , plasma was treated with trichloroacetic acid and radioactivity was determined in the protein-free supernatant obtained after centrifuging. There was a progressive

Table 1. *Analysis of protein samples*

Methods for the preparation of the dried protein samples and for their analysis are given in the text. Collagen figures are derived from the amount of hydroxyproline $\times 6.9$. Samples analysed were obtained from young adult rats.

Sample	Protein (%)	Nitrogen (%)	Collagen (%)	Phosphorus (%)
Brain and cord residue	100.2	12	1.5	0.4
Proteolipid protein	—	—	0	0.4
Sciatic nerve residue	—	7.2	11	0.4
Muscle residue	97.5	14	3.8	0.1
Tendon	88	13	100	0.02
Kidney residue	97	—	—	—

Fig. 3. Levels of radioactivity in plasma and whole blood of adult rats (A) and neonatal rats (B) at various intervals after the administration of radioactive glycine. Radioactivity in plasma (●) and whole blood (○) was determined by Geiger-Müller counting as described in the text. The extrapolation of the curves beyond the last readings shown is based on further measurements made at 48 and 72 days for young rats and 42 and 72 days for adults.

loss of radioactivity from the blood of young and adult rats, the rate being greater for plasma than for whole blood (Fig. 3). No radioactivity was detected in the protein-free plasma extract 8 days after injection in the neonatal animals, and only 3% of the plasma radioactivity was in this form 19 hr. after injection. In the adult animals protein-free plasma extract was free of detectable radioactivity 24 hr. after injection of the glycine. These results indicate that circulating radioactive precursors rapidly disappear from the blood (see also Greenberg & Winnick, 1948; Henriques, Henriques & Neuberger, 1955), and, therefore, the present investigation is not complicated as was the ^{32}P study by this persistence.

Proteins of the central nervous system. Rats were killed at intervals after injection with labelled glycine, and the brain and spinal cord extracted with chloroform-methanol (2:1, v/v). This initial treatment (stage 1) separated lipid and most of the proteolipid of the central nervous system from an insoluble residue [residue (A), Fig. 1]. After removal of a sample of the washed chloroform-

methanol extract, the remainder was mixed with a synthetic upper phase containing potassium citrate (G. R. Webster & J. Folch, in preparation) to dissociate the proteolipid (stage 4, Fig. 1). The proteolipid protein (F) obtained by this latter treatment was washed with different solvents (see above) and analysed for nitrogen and phosphorus (Table 1). A sample was also refluxed with 6N-hydrochloric acid for 6 hr. and the hydrolysate separated by paper chromatography; it contained at least 18 different amino acids, confirming that the substance was a protein.

Analysis of the chloroform-methanol-insoluble residue from the brain and cord indicated that this consisted almost entirely of protein. Traces of carbohydrate and phosphorus and the equivalent of about 1.5% of collagen were also present in the protein residue (Table 1). Some of the chloroform-methanol-insoluble residue was incubated with trypsin (LeBaron & Folch, 1956) and the trypsin-resistant protein residue prepared.

Persistence of radioactivity in the proteins of the central nervous system of the rat. The ^{14}C content of proteolipid protein was determined by two techniques. The first and more accurate method (method 1) depended on the difference between the radioactivity of an extract containing chloroform-methanol-soluble lipid plus proteolipid and the radioactivity of the same extract after removal of proteolipid protein. In some cases radioactivity of the separated proteolipid protein was determined directly (method 2). Difficulties in producing a fine powder suitable for scintillation counting account for any differences in radioactivity content found by the two methods (Tables 2 and 3).

The proteolipid protein that was obtained from rats, which had been injected with $[1-^{14}\text{C}]$ glycine when 11 days old, retained its radioactivity for at least 250 days (Table 2 and Fig. 4). After injection of labelled glycine into adult rats much less ^{14}C was incorporated into the proteolipid protein of the central nervous system. Thus a day after injection the corrected specific activities of proteolipid protein ($\mu\mu\text{C}/\text{mg.}$ of protein) were 373 and 2.5 for neonatal and adult rats respectively. Further, the results suggest that the labelled proteolipid protein

Table 2. *Radioactivity of proteins of the central nervous system at intervals after injection of [1-¹⁴C]glycine into 11-day-old rats*

Rats previously injected with 5 μ C of [1-¹⁴C]glycine were killed at intervals up to 250 days. The preparation of the various samples is described in the text. Radioactivity of the 'protein' residue counted at infinite thickness by thin end-window Geiger-Müller counter is expressed in arbitrary units equivalent to counts/min. of sample. Radioactivity of other protein fractions has been determined by scintillation counting as described in the text. Figures for radioactivity should be multiplied by 0.6 to give the equivalent of a dose of 10 μ C of [1-¹⁴C]glycine/100 g. body wt.

Radioactivity of total central nervous system									
Time after injection	Body wt. when killed (g.)	Wet wt. of brain and cord (g.)	Wt. of washed chloroform-insoluble residue (mg.)	Whole protein residue less proteolipid protein [residue (A)]			Proteolipid protein		
				Determined by Geiger-Müller counting (counts/min.)	Whole untreated residue determined by scintillation counting	Whole alkali-stable residue (μ C)	Whole trypsin-resistant protein residue (μ C)	Determined by difference in radioactivity of lipid extracts (D) and (E), method 1 (μ C)	Determined directly by counting suspension (F) in gel, method 2 (μ C)
2 hr.	25	1.19	200	204	27 800	—	—	—	—
18	30	1.21	160	200	17 000	—	1750	620	—
19	30	1.22	140	178	11 400	—	1060	470	—
3 days	28	1.37	80*	102	8 100	640	1200	—	—
9	55	1.48	127	—	10 700	—	780	350	155
10	52	1.76	144	110	—	—	780	450	—
23	134	2.0	208	71	5 320	—	780	270	290
42	168	2.16	291	47	2 380	—	444	220	400
72	380	2.41	223	19	1 030	—	185	405	—
126	390	2.54	251	15	670	160	122	260	280
189	280	2.71	268	6	300	160	64	290	190
250	258	2.59	264	3	228	127	—	—	—

* This result is low owing to poor recovery.

Table 3. *Radioactivity of proteins of the central nervous system at intervals after injection of [1-¹⁴C]glycine into mature rats*

Six adult female rats of about 350 g. were given 16.6 μ C of [1-¹⁴C]glycine by intraperitoneal injection. For details of determination of radioactivity see Table 2 and text. Figures for radioactivity should be multiplied by 2.1 to give the equivalent of a dose of 10 μ C of [1-¹⁴C]glycine/100 g. body wt.

Time after injection (days)	Wet wt. of brain and cord (g.)	Wt. of washed chloroform-insoluble residue (mg.)	Radioactivity of total central nervous system					
			Whole protein residue less proteolipid protein [residue (A)]			Proteolipid protein		
			Determined by Geiger-Müller counting (counts/min.)	Whole untreated residue determined by scintillation counting ($\mu\mu\text{C}$)	Whole alkali-stable residue ($\mu\mu\text{C}$)	Whole trypsin-resistant protein residue (C) ($\mu\mu\text{C}$)	Determined by difference in radioactivity of lipid extracts (D) and (E), method 1 ($\mu\mu\text{C}$)	Determined directly by counting suspension (F) in gel, method 2 ($\mu\mu\text{C}$)
1	2.98	309	43	3520	—	60	—	29
4	2.63	211	18	1770	—	232	32	23
18	2.76	246	14	1120	—	210	20	34
49	2.87	244	4	590	18	57	78	44
190	2.69	259	1	78	0	0	5	4
197	3.15	280	2	50	0.1	35	12	8

of the central nervous system in adult rats undergoes slow turnover (Table 3). Clouet & Richter (1959) found that there was relatively slow incorporation of ^{35}S into the cerebral proteolipid protein of 28-day-old rats.

The major proportion of brain and spinal cord proteins (excluding proteolipid protein and collagen) are in a state of dynamic metabolism. After injection of $[1-^{14}\text{C}]\text{glycine}$ into both neonatal and adult groups of rats relatively large amounts of ^{14}C

are incorporated into the central-nervous-system proteins [residue (B), Fig. 1]. The rate of loss of labelled protein from the central nervous system of both groups is exponential (see Fig. 4 and Tables 2 and 3) with a half-life of about 22 days. Some conservation of radioactivity in the protein residue of rats, which had been injected with $[1-^{14}\text{C}]\text{glycine}$ when 11 days old, was associated with the alkali-insoluble protein, and thus this apparent persistence of about 1% of the initial radioactivity can be ascribed to retention of ^{14}C in collagen of the central nervous system.

Persistence of radioactivity in the proteins of the peripheral nervous system. Radioactivity of chloroform-methanol-insoluble protein of sciatic nerve has been determined, at intervals after injection of neonatal and adult rats, by scintillation counting of the dry protein powder in suspension. Since it is not practicable to dissect out whole sciatic nerve in a reproducible way for different animals, particularly young rats, results for nerve are recorded as specific activities. The specific activity is expressed as radioactivity/mg. of dried protein residue. Specific activities are further corrected to a dose of $10\text{ }\mu\text{C}/100\text{ g. body wt.}$ (to facilitate comparison between neonatal and adult groups of rats), and the results for the neonatal group of rats are also corrected for the dilution effect of growth. This latter correction is based on the assumption that increase in nerve protein parallels increase in body weight. Such an assumption seems reasonable as the increases in wet weight of sciatic nerve recovered are proportional to body weight during development.

If the results obtained for the neonatal and adult rats are compared two important differences emerge. First, much less radioactive glycine is

Fig. 4. Levels of radioactivity in proteins of the central nervous system of rats at various intervals after the administration of $[1-^{14}\text{C}]\text{glycine}$. Radioactivity of total central-nervous-system protein other than proteolipid protein is for convenience divided by 10 (●). Persistence of radioactivity in whole brain and cord proteolipid protein (×) is indicated by the straight line calculated by the method of least squares. The line includes values from 10 to 250 days after injection of labelled glycine. Results are expressed in terms of a dose of $5\text{ }\mu\text{C}/[1-^{14}\text{C}]\text{glycine}$ injected into 11-day-old rats of a mean body weight of 30 g.

Table 4. *Persistence of radioactivity in the protein residue from the sciatic nerve of young and adult groups of rats at intervals after an injection of $[1-^{14}\text{C}]\text{glycine}$*

Methods for the preparation and determination of sample radioactivity are given in the text. Specific activity is given as radioactivity $\mu\text{C}/\text{mg.}$ of protein residue corrected for a dose of $10\text{ }\mu\text{C}$ of $[1-^{14}\text{C}]\text{glycine}/100\text{ g. body wt.}$ Specific activities for young rats are also corrected for the dilution effect of growth.

Rats injected with $5\text{ }\mu\text{C}$ of $[1-^{14}\text{C}]\text{glycine}$ when 11 days old

Rats injected with $16.6\text{ }\mu\text{C}$ of $[1-^{14}\text{C}]\text{glycine}$ when adult

Time after injection (days)	Wt. of sciatic nerve (mg.)	Wt. of chloroform-methanol-insoluble protein residue (mg.)		Specific activity corrected for growth and dose	Time after injection (days)	Wt. of sciatic nerve (mg.)	Wt. of chloroform-methanol-insoluble protein residue (mg.)		Specific activity corrected for dose
1	16	1.1		265	1	134	18.3		30
42	60	2		87	4	115	11		5.7
72	131	11		254	18	139	15.7		12.0 (1.6)*
126	166	18		140	49	123	7.4		1.9
189	92	12		210 (63)*	190	115	17.8		2.3 (0.4)*
250	90	10		67 (46)*	197	140	14.4		1.3 (0.6)*

* Radioactivity of alkali-insoluble protein residue of sciatic nerve.

incorporated into the nerve proteins of adult compared to the 11-day-old rats. Secondly, the specific activity of sciatic-nerve protein in the mature series of rats declines during the period studied, and only about 7% of the original activity remains 190–197 days after injection. In those rats injected with [^{14}C]glycine when 11 days old, 25% of the original radioactivity remains 250 days after injection (Table 4).

Persistence of radioactivity in the proteins of muscle and tendon. Radioactivity of muscle and tendon protein was determined by scintillation counting of a suspension in a gel. Since it is impracticable to express the radioactivity as total body muscle or tendon protein activity the results

Table 5. *Radioactivity of tendon and muscle proteins at intervals after injection of [^{14}C]glycine into 11-day-old rats*

Methods for extraction and for the determination of radioactivity by scintillation counting are described in the text. The results are given as specific activities corrected for the dilution effect of growth and for a dose of $10\text{ }\mu\text{C}$ of [^{14}C]glycine/100 g. body wt. All specific activities are recorded as radioactivity $\mu\text{C}/\text{mg.}$ of dry unhydrolysed protein.

Time after injection of $5\text{ }\mu\text{C}$ of [^{14}C]glycine	Radioactivity in collagen		Radioactivity in muscle protein	
	Protein residue specific activity	Alkali-stable residue specific activity	Protein residue specific activity	Alkali-stable residue specific activity
19 hr.	104	58	17	12
3 days	220	185	65	14
23	71	100	56	12
42	53	54	35	5
72	20	27	35	10
126	37	27	36	10
189	37	48	36	13
250	52	43	13	6

Table 6. *Radioactivity of tendon and muscle proteins at intervals after injection of [^{14}C]glycine into mature rats*

For details see Table 5.				
Time after injection of $16\text{--}6\text{ }\mu\text{C}$ of [^{14}C]glycine (days)	Radioactivity in collagen		Radioactivity in muscle protein	
	Protein residue specific activity	Alkali-stable residue specific activity	Protein residue specific activity	Alkali-stable residue specific activity
1	1.93	1.6	5.5	0.6
4	1.64	—	9.6	7.4
18	0.73	—	17.0	4.9
49	0.45	—	8.7	3.3
190	0.26	0.38	0.75	0
197	0.15	0.12	2.1	0.1

are shown as specific activities corrected for a dose of [^{14}C]glycine of $10\text{ }\mu\text{C}/100\text{ g.}$ body wt. and for the dilution effect of growth in the developing rats (Table 5). This later correction is based on the assumption that accumulation of unlabelled protein parallels increase of body weight (Neuberger, Perrone & Slack, 1951). Even if no corrections are applied the same general picture emerges. Radioactivity was also determined in the alkali-insoluble muscle and tendon residues. The specific activities were calculated in terms of the weight of original material taken before alkali digestion. Table 5 shows that there is rapid incorporation of glycine into muscle protein and particularly into the tendon of the developing rats. There is loss of radioactivity from both proteins up to about 23 days after injection of the precursor into neonatal rats, after that the level of radioactivity remains with little change until the end of the experiment (250 days). Almost all the radioactivity incorporated into the tendon is located in alkali-stable protein, which is largely collagen. In the muscle protein the [^{14}C]glycine is incorporated into both alkali-soluble and alkali-insoluble protein and the persisting radioactivity remains in both fractions for at least 250 days.

Much less radioactivity is incorporated into muscle and tendon protein of the adult rats. Further, a high proportion of this radioactivity is found in the alkali-soluble muscle protein (Table 6). The radioactivity is lost only slowly from the labelled muscle and tendon protein.

Fate of radioactivity incorporated into kidney proteins

Radioactivity was also determined in the kidney proteins at intervals after the injection of [^{14}C]glycine into 11-day-old and adult rats (Table 7). The results in Table 7 show the total radioactivity of the protein from one kidney of each rat. After an initial period (4–10 days) in both neonatal and adult groups of rats there is a steady loss of ^{14}C from the kidney proteins. The small amount of radioactivity found at the end of the experiment in the proteins of the developing rats is almost entirely restricted to the alkali-insoluble protein residue (collagen) from the kidney.

DISCUSSION

The classical studies of Schoenheimer (1946) led to the discovery of the dynamic state of body proteins and other constituents. Subsequently many workers have confirmed and extended Schoenheimer's original concept of rapid protein metabolism. Thus it has been shown in experiments *in vivo* that labelled amino acids can be readily incorporated into brain and other body proteins

Table 7. *Radioactivity of kidney lipid-free residue at intervals after injection of [1-¹⁴C]glycine into neonatal and mature rats*

Kidney was extracted with chloroform-methanol (2:1, v/v) and after filtration the residue was successively washed with ethanol, water and ethanol. Radioactivity of the total dried residue (about 97% of protein) was determined by scintillation counting, and the results are corrected to the equivalent of a dose of 10 μ C of [1-¹⁴C]-glycine/100 g. body wt. Neonatal and mature rats were injected with 5 and 16.6 μ C of [1-¹⁴C]glycine respectively.

Neonatal rats			Mature rats		
Time after injection (days)	Dry wt. of residue (mg.)	Radioactivity (μ C)	Time after injection (days)	Dry wt. of residue (mg.)	Radioactivity (μ C)
1	18.1	2840	1	161.8	10 700
1	30.7	1270	4	125.5	13 700
3	11.3	1140	18	245.4	1 700
9	31.2	1305	49	179.6	597
10	11.2	1650	190	222.2	258
23	46.4	625	197	260.9	354
42	132.7	630	—	—	—
72	211.4	150	—	—	—
126	178.2	130	—	—	—
250	118.4	77	—	—	—

(Greenberg & Winnick, 1948; Vladimirov & Urinson, 1957; Kravchinsky & Silich, 1957; Furst, Lajtha & Waelsch, 1958; Clouet & Richter, 1959; Lajtha, Berl & Waelsch, 1959). Although it is recognized that brain proteins are not homogeneous and the range of turnover rates of brain proteins is very wide and possibly includes less active or completely inactive fractions (Clouet & Richter, 1959; Lajtha, 1959), these short-term studies were taken to indicate that adult rat-brain and mouse-brain proteins as a whole have a mean half life of between 14 and 27 days.

Although very slow turnover rates have been reported for part of muscle protein (Neuberger *et al.* 1951; Thompson & Ballou, 1954) and for collagen (Neuberger & Slack, 1953) it does not appear that any attention has been paid to studying the retention of labelled neural proteins for long periods. Neither has there been any systematic comparison between long-term protein metabolism in young and adult animals.

The present work indicates that neural and muscle proteins are composed of groups of both metabolically active and inactive protein. The definition of metabolically inactive or stable proteins used in this present work applies to an experiment lasting only 250 days. It is therefore possible that very slow turnover may occur during the lifetime of the rat or equally that there may be complete metabolic inertness of certain protein fractions. Another possibility that must be considered is that internal exchange and re-utilization of radioactive amino acid occurs *in situ*; however, if this was happening it is probable that a slow loss of radioactivity would become apparent. Further, if recycling of degradation products of radioactive protein was occurring such a process must be restricted to the proteolipid proteins, for

there was a continuous loss of radioactivity from other proteins of the central nervous system.

Protein metabolism of the central nervous system. It has been shown that metabolism of proteins, like that of lipids, can be divided into metabolically active and inactive pools. Those proteins which are metabolically active comprise the major part of the proteins of the central nervous system and include the trypsin-resistant protein residue part of which probably comprises the chemically inert neurokeratin (LeBaron & Folch, 1956). The mean turnover rates of these groups of proteins are indicated in Table 8 together with those obtained by other authors. In the present work the turnover rates for protein residues of both young and adult groups of rats are the same and in reasonable agreement with the half-lives reported by other authors for adult rats and mice. In these latter experiments [³⁵S]methionine, [¹⁴C]leucine or lysine was injected into animals and the half-life was calculated from the degree of incorporation into brain proteins. The faster turnover rates obtained (Lajtha, 1959; Lajtha, Furst, Gerstein & Waelsch, 1957) for very young mice may be due, as suggested by the authors, to predominant incorporation into rapidly metabolizing brain-protein fractions. Another factor influencing the rate of incorporation of amino acids into brain protein is that in young animals structural, relatively inert, proteins are being synthesized and that turnover rates determined by extrapolation will thereby be artificially increased. These limitations of the short-term metabolism experiments are, however, clearly recognized by Clouet & Richter (1959) and by Lajtha (1959).

In those animals injected with [1-¹⁴C]glycine when 11 days old, there is a persistence of radioactive proteolipid protein lasting for at least

Table 8. *Mean turnover rates of labelled proteins of the central nervous system*

Mean turnover rates of whole brain and cord (1) were obtained directly from the biological decay curve. Other mean turnover rates are for brain proteins and were calculated from the incorporation curves obtained in short-term experiments.

Animal	Age (days)	Amino acid injected	Duration of expt.	Protein preparation	Mean half-life (days)	Reference
Rat	11	[1- ¹⁴ C]Glycine	250 days	Whole protein central nervous system residue	22	1
				Trypsin-resistant residue	22	1
Rat	Adult	[1- ¹⁴ C]Glycine	250 days	Whole protein central nervous system residue	22	1
				Trypsin-resistant residue	22	1
Rat	Young (36-77 g.)	[³⁵ S]Methionine	20-60 min.	Mixed brain proteins	14	2
Mouse	Newborn	[¹⁴ C]Leucine	60 min.	Mixed brain proteins	4.1	3
Mouse	Adult	[¹⁴ C]Leucine	60 min.	Mixed brain proteins	27	3
Mouse	8	[¹⁴ C]Lysine	45 min.	Mixed brain proteins	2.4	4
Mouse	Adult	[¹⁴ C]Lysine	60 min.	Mixed brain proteins	15.2	4

1, This paper; 2, Gaitonde & Richter (1956); 3, Lajtha (1959); 4, Lajtha, Furst, Gerstein & Walsch (1957).

250 days. This persistence indicates a quite remarkable degree of metabolic inertness compared with that of other brain and cord proteins. In addition, a relatively small amount of radioactivity persisting in the protein residue of the central nervous system has been shown to be probably due to the retention of ¹⁴C in the collagen present in the organ.

In contrast with these findings in the young rats, much less radioactive glycine is incorporated into the relatively larger proteolipid-protein content of the adult rat brain and cord. Such radioactivity as is incorporated undergoes slow turnover and may well be associated with non-permanent cell or particle membranes. Similarly, there is little retention of radioactivity in other proteins from the central nervous system of adult rats injected with [1-¹⁴C]glycine. These results support the hypothesis that proteolipid protein is an integral part of the myelin sheath and, if inert, once labelled during development the radioactivity would persist throughout life in the healthy normal animal. It would also follow that if proteolipid protein was predominantly inert there would be a failure to incorporate and retain radioactive glycine in this protein in the adult animal.

Protein metabolism of the peripheral nervous system. The results shown in Table 4 indicate that a larger fraction of nerve-protein residue retains the incorporated radioactivity than is retained in the protein residue of the central nervous system. This persistent radioactivity is not wholly due to the labelling of metabolically inert collagen or elastin present in the nerve. Peripheral nerve differs from the central nervous system in that only traces of proteolipid soluble in chloroform-

methanol can be extracted from nerve (Folch *et al.* 1958; Finean, Hawthorne & Patterson, 1957). It is therefore probable that the protein residue obtained from the sciatic nerve contains relatively large quantities of myelin-sheath protein and that it is this protein fraction which accounts for the conservation of stable ¹⁴C in the residue.

In the adult rat loss of radioactivity from the residue of sciatic nerve insoluble in chloroform-methanol is comparable to the loss of radioactivity from the protein residue of the central nervous system. This difference in the behaviour of nerve protein in the young and adult group of animals suggests that in the adult only non-structural proteins incorporate radioactive glycine and that the structural proteins remain metabolically inert and inaccessible to the precursor.

Protein metabolism of muscle and collagen. It was first recognized by Sprinson & Rittenburg (1949) that although proteins of plasma and some of the internal organs undergo rapid metabolism (see Greenberg & Winnick, 1948, and this paper) other proteins of muscle, skin, etc. are relatively inert metabolically. Direct experimental evidence for this concept was reported by Neuberger and his associates (Neuberger, 1955; Henriques *et al.* 1955) and by Thompson & Ballou (1954, 1956). The present experiments differ in some respects from those of these earlier workers. Thus not only have more animals been used in experiments over a considerably extended period but some attempt has also been made to divide the protein into alkali-stable ('collagen') and alkali-soluble fractions.

Neuberger *et al.* (1951) showed that much less [¹⁴C]glycine was incorporated into collagen of old

compared with young rats and that there was retention of the radioactivity incorporated into the collagen of the young animals. These findings were confirmed in the present investigation. It also appears that collagen, like other body constituents, can be divided into metabolically active and inactive groups. Thus in young animals there was turnover of incorporated radioactive carbon until 42 days after injection. After that, between 42 and 250 days, there was almost complete retention of radioactivity. Similarly, [1-¹⁴C]glycine was incorporated into alkali-soluble and alkali-insoluble (collagen) muscle proteins of neonatal rats. There was little turnover of labelled muscle collagen, but other muscle proteins lost about half the incorporated ¹⁴C up to 42 days after injection. From 42 to 250 days after injection there was a retention of radioactivity. These findings suggest that part of the actomyosin may be metabolically inert.

After injection of labelled glycine into adult rats a relatively small amount of ¹⁴C was incorporated into collagen and muscle proteins. There was slow turnover of the labelled collagen and no indication of the metabolic stability of the major part of the collagen, which can only be demonstrated by introducing radioactive glycine into developing rats. Up to 18 days after injection there was an increase in radioactivity incorporated into the muscle protein of adult rats. Recently Dreyfus, Kruh & Schapira (1960) have demonstrated a similar retention of ¹⁴C-labelled myosin in adult rats injected with glycine uniformly labelled with ¹⁴C. As these authors suggest, the persistence of radioactivity in the myosin may only be apparent, for they demonstrated that free labelled glycine remains in muscle for several weeks after injection. Thus it is probable that the increase in radioactivity of muscle protein of adult rats up to 18 days after injection, reported in the present paper, was due to continued synthesis of new labelled protein. This possibility is supported by the finding of Dreyfus *et al.* (1960) that there is an immediate and progressive loss of radioactivity from myosin in rats fed on a high-protein diet when the free labelled glycine of muscle would be expected to be rapidly replaced by non-radioactive amino acid. In the present study the fate of radioactive muscle protein was followed for a period up to 196 days after the initial injection. From 18 days after injection to the end of the experiment, there was a slow loss of ¹⁴C from the muscle protein. Unfortunately the experiments of Dreyfus *et al.* (1960), suggesting that adult rat myosin is relatively inert, were continued for only 55 days and slow turnover of myosin was only observed by them in rats fed a high-protein diet. Metabolic stability of part of the muscle protein can, however, be demonstrated by

incorporating [1-¹⁴C]glycine into developing rats (see above).

The present findings therefore fit in with the general hypothesis that body constituents can be divided into metabolically active and metabolically inert (structural) compartments. Wholly metabolically active constituents are typified by the plasma or kidney proteins or by the lipids of some of the internal organs. At the other extreme are deoxyribonucleic acid (Hecht & Potter, 1958), haemoglobin (Shemin & Rittenberg, 1946; Muir, Neuberger & Perrone, 1952) and some of the brain lipids (Davison & Dobbing, 1960*a*) which are probably metabolically inert. Other body constituents can be divided into both stable and metabolically active compartments. Thus in the present work a proportion of the muscle and collagen proteins and some of the proteins of the central nervous system have been shown to be stable, the remainder undergoing dynamic metabolism.

SUMMARY

1. After the injection of [1-¹⁴C]glycine into neonatal and mature rats it has been shown that proteins of the central and peripheral nervous system, of muscle and tendon can be divided into metabolically active and inactive groups.

2. Metabolic inertness of proteolipid protein of the central nervous system is suggested by the retention of radioactivity in this protein for at least 250 days after injection of [1-¹⁴C]glycine into neonatal rats.

3. Relatively little radioactivity is incorporated into the proteolipid protein of adult rats injected with labelled glycine and the radioactive proteolipid protein undergoes slow turnover.

4. Proteins of the central nervous system, other than collagen and proteolipid, in both young and adults have been shown to be metabolically active with turnover rates comparable with those of plasma and kidney proteins.

5. Radioactivity persists for at least 250 days in sciatic nerve collagen and other proteins after injection of 11-day-old rats, but in adult rats nerve proteins undergo dynamic metabolism.

6. Similar metabolic inertness has been observed in some muscle and tendon proteins after injection of neonatal but not mature rats.

7. It is suggested that the metabolic inertness of certain neural, muscle and tendon proteins is associated with stable structural elements such as the myelin sheath, collagen and actomyosin. This metabolic stability can only be demonstrated by incorporation of radioactive isotopes into these proteins during development.

The author wishes to thank Miss Jennifer Vogt for her valuable technical assistance.

REFERENCES

- Aldridge, W. N. (1957). *Biochem. J.* **67**, 423.
- Clouet, D. H. & Richter, D. (1959). *J. Neurochem.* **3**, 219.
- Davison, A. N. & Dobbing, J. (1959). *Biochem. J.* **73**, 701.
- Davison, A. N. & Dobbing, J. (1960a). *Biochem. J.* **75**, 565.
- Davison, A. N. & Dobbing, J. (1960b). *Biochem. J.* **75**, 571.
- Davison, A. N., Dobbing, J., Morgan, R. S. & Payling Wright, G. (1959). *Lancet*, i, 658.
- Davison, A. N., Morgan, R. S., Wajda, M. & Payling Wright, G. (1959). *J. Neurochem.* **4**, 360.
- Davison, A. N. & Wajda, M. (1959). *J. Neurochem.* **4**, 353.
- Dreyfus, J. C., Kruh, J. & Schapira, G. (1960). *Biochem. J.* **75**, 574.
- Finean, J. B., Hawthorne, J. N. & Patterson, J. D. E. (1957). *J. Neurochem.* **1**, 256.
- Finean, J. B. & Robertson, J. D. (1958). *Brit. med. Bull.* **14**, 267.
- Folch, J. & Lees, M. (1951). *J. biol. Chem.* **191**, 807.
- Folch, J., Lees, M. & Carr, S. (1958). *Exp. Cell Res.* (Suppl.), **5**, 58.
- Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957). *J. biol. Chem.* **226**, 497.
- Furst, S., Lajtha, A. & Waelsch, H. (1958). *J. Neurochem.* **2**, 216.
- Gaitonde, M. K. & Richter, D. (1956). *Proc. Roy. Soc. B*, **145**, 83.
- Greenberg, D. M. & Winnick, T. (1948). *J. biol. Chem.* **173**, 199.
- Hecht, L. I. & Potter, V. R. (1958). *Cancer Res.* **18**, 186.
- Henriques, O. B., Henriques, S. B. & Neuberger, A. (1955). *Biochem. J.* **60**, 409.
- Koenig, H. (1959). *J. Neurochem.* **4**, 93.
- Kravchinsky, E. M. & Silich, T. P. (1957). *Ukr. biochem. J.* **29**, 25.
- Lajtha, A. (1959). *J. Neurochem.* **3**, 358.
- Lajtha, A., Berl, S. & Waelsch, H. (1959). *J. Neurochem.* **3**, 322.
- Lajtha, A., Furst, S., Gerstein, A. & Waelsch, H. (1957). *J. Neurochem.* **1**, 289.
- LeBaron, F. N. & Folch, J. (1956). *J. Neurochem.* **1**, 101.
- Levy, A. L. & Chung, D. (1953). *Analyt. Chem.* **25**, 396.
- Lowry, O. H., Gilligan, D. R. & Katersky, E. M. (1941). *J. biol. Chem.* **139**, 795.
- Muir, H. M., Neuberger, A. & Perrone, J. C. (1952). *Biochem. J.* **52**, 87.
- Neuberger, A. (1955). *Symp. Soc. exp. Biol.* **9**, 72.
- Neuberger, A., Perrone, J. C. & Slack, H. G. B. (1951). *Biochem. J.* **49**, 199.
- Neuberger, A. & Slack, H. G. B. (1953). *Biochem. J.* **53**, 47.
- Neuman, R. E. & Logan, M. A. (1950). *J. biol. Chem.* **184**, 299.
- Robinson, H. W. & Hogden, C. G. (1940). *J. biol. Chem.* **135**, 709.
- Schoenheimer, R. (1946). *The Dynamic State of Body Constituents*. Cambridge, Mass.: Harvard University Press.
- Shemin, D. & Rittenberg, D. (1946). *J. biol. Chem.* **166**, 627.
- Sprinson, D. B. & Rittenberg, D. (1949). *J. biol. Chem.* **180**, 715.
- Stitch, S. R. (1959). *Biochem. J.* **73**, 287.
- Thompson, R. C. & Ballou, J. E. (1954). *J. biol. Chem.* **208**, 883.
- Thompson, R. C. & Ballou, J. E. (1956). *J. biol. Chem.* **223**, 795.
- Uzman, L. L. (1958). *Arch. Biochem. Biophys.* **76**, 474.
- Uzman, L. L. & Rosen, H. (1958). *Arch. Biochem. Biophys.* **76**, 490.
- Vladimirov, G. E. & Urinson, A. P. (1957). *Biokhimiya*, **22**, 665.

Biochem. J. (1961) **78**, 282

The Location of Carbohydrases in the Digestive Tract of the Pig

By A. DAHLQVIST

Department of Physiological Chemistry, University of Lund, Lund, Sweden

(Received 18 July 1960)

Little is known about the locations in the mammalian intestine at which hydrolysis of disaccharides and polysaccharides occurs.

In a recent investigation of intestinal digestion and absorption in man, Borgström, Dahlqvist, Lundh & Sjövall (1957) observed that the invertase activity (in unpublished experiments we have demonstrated that the maltase and lactase activities are distributed similarly) of the intestinal contents, which was weak throughout the whole small intestine, was maximal in the lower part of the small intestine (lower jejunum and ileum). In spite of this, the absorption of lactose occurred in

the upper part of the small intestine (duodenum and upper jejunum), where no disaccharidase activity could be demonstrated in the intestinal contents (Ammon & Henning, 1956; Borgström *et al.* 1957). The hydrolysis of disaccharides during their absorption is catalysed by enzymes apparently situated inside the cells of the intestinal mucosa (Borgström *et al.* 1957). Little information is, however, available about the relative disaccharidase activity of the mucosa of different parts of the small intestine.

Amylase, in contrast to the disaccharidases, is mainly secreted into the intestinal lumen in the