with time in this ratio for the metabolism of ^{32}P when injected into developing or mature rabbits. This work is therefore fully consistent with the hypothesis that the lipids of the myelin sheath are metabolically inert. Furthermore, it ought to be possible, by more refined anatomical and biochemical techniques, to establish the hypothesis in even greater detail.

SUMMARY

1. ^{32}P has been injected into developing and adult rabbits and its fate investigated in the phospholipids of white and grey matter of brain.

2. Those phospholipids which have been found to persist are predominantly located in the white matter.

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REFERENCES

- Ansell, G. B. & Dohmen, H. (1957). *J. Neurochem.* **2**, 1.
 Ansell, G. B. & Spanner, S. (1959). *Biochem. J.* **73**, 3 p.
 Davison, A. N. & Dobbing, J. (1959). *Biochem. J.* **73**, 701.
 Davison, A. N. & Dobbing, J. (1960). *Biochem. J.* **75**, 565.
 Davison, A. N., Dobbing, J., Morgan, R. S. & Wright, G. P. (1959). *Lancet*, i, 658.
 Davison, A. N., Morgan, R. S., Wajda, M. & Wright, G. P. (1959). *J. Neurochem.* **4**, 360.
 Fries, B. A. & Chaikoff, I. L. (1941 a). *J. biol. Chem.* **141**, 469.
 Fries, B. A. & Chaikoff, I. L. (1941 b). *J. biol. Chem.* **141**, 479.
 Miani, N. & Bucciante, G. (1957). *Atti Soc. med.-chir. Padova*, **34**, 5.

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Metabolism of Myosin and Life Time of Myofibrils

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The concept of the dynamic state of body proteins is now generally admitted, but recent work has made some reappraisal necessary. Proteins may undergo three types of metabolic patterns. Some turn over at a rather fast rate, but the turnover is the result of a secretion out of the cells: such is true of digestive enzymes or of the plasma proteins as studied by Miller, Bly, Watson & Bayle (1951). Others remain in the cells and undergo turnover; Dreyfus, Schapira & Kruh (1956) have shown that this is so with muscle aldolase. On the other hand Shemin & Rittenberg (1945) demonstrated that haemoglobin does not turn over during the life of the cell. Later this concept was applied by Neuberger, Perrone & Slack (1951) and by Robertson (1952) to collagen, which might remain inert during the whole life of the animal. Thompson & Ballou (1956) have emphasized the quantitative importance of apparently inert proteins. Shemin & Rittenberg (1944) showed that the muscle proteins can be divided into two groups, one of which is rapidly metabolized and the other slowly metabolized. Myosin belongs to the slowly metabolized group (Bidinost, 1951).

Neuberger (1952) suggested that 'the metabolic activity of actin or myosin in the muscle fibre, or

that of collagen, for example, is not a continuous function, but varies in a discontinuous manner and is dependent on morphological changes of the structures with which these proteins are associated'. This idea has never been experimentally checked for muscle proteins. In this work we have tried to determine whether myosin is in a dynamic equilibrium or whether it is inert enough to permit the determination of the life span of the cell or of a part of the cell. A preliminary note has been previously published (Dreyfus, Kruh & Schapira, 1957).

METHODS

Injection of radioactive glycine. White male rats of pure Wistar strain, weighing 200–250 g., fed with an identical diet, were used in each series of experiments. In these conditions, the specific activity of radioactive proteins from two animals given the same diet did not differ by more than 15%. The animals were given two types of diet, a 'standard' diet and a 'protein-rich' diet. The standard diet was a commercial one containing (%): oats (3), corn (5), wheat (30), molasses (2), wheat and barley seeds (8), lucerne flour (4), defatted peanut meal (5), defatted soya meal (11), defatted flax meal (5), skimmed-milk powder (3), meat flour (4), fish flour (4) and brewer's yeast (3), together with salts and vitamins. This standard diet contained approx. 25% of protein. The 'protein-rich' diet was pre-

pared by mixing 73 parts of the standard diet with 27 parts of casein, and it contained 45% of protein. The animals were starved for 16 hr. before and 6 hr. after the injection of radioactive glycine. Glycine uniformly labelled with ^{14}C was injected intraperitoneally, at a dosage of $5\text{ }\mu\text{C}$ ($100\text{ }\mu\text{g.}$)/100 g. body wt. In one experiment [^{14}C]valine was used. The animals were killed by bleeding after chloral anaesthesia; tissues were immediately excised and put in ice.

Muscle proteins. Muscles were crushed in a Latapie's apparatus. About 30 g. was obtained from each rat. Myosin was prepared according to Szent-Gyorgyi (1951); its purity was ascertained by moving-boundary electrophoresis. The absence of actomyosin was always checked by the absence of fall in viscosity under the action of adenosine triphosphate. Myosin was precipitated by 20% trichloroacetic acid. Aldolase was crystallized three times by the method of Warburg & Christian (1943) modified by Taylor, Green & Cori (1948). The purity of the protein was verified in preliminary experiments by electrophoresis and by the absence of activity of phosphorylase, phosphoglucosomutase, phosphoglyceraldehyde dehydrogenase and α -glycerophosphate dehydrogenase. Water-soluble proteins were prepared by extraction of crushed muscle with 4 vol. of water and, after filtration, by precipitation of the protein with 1 vol. of 20% trichloroacetic acid.

Myofibrils. Myofibrils were prepared according to Perry (1953). Muscles were crushed with Latapie's apparatus and then homogenized in a Waring Blender in borate buffer during 90 sec. in the cold room. Myofibrils were isolated and purified by five centrifugings at 600 g for 20 min., the upper part of the residue being collected each time, then by centrifuging at 300 g for 2 min., at which speed impurities are deposited. Microscopic examinations were made and the proteins were precipitated by 1 vol. of 20% trichloroacetic acid, then dissolved again in N-NaOH and reprecipitated by 20% trichloroacetic acid.

Globin. The red cells were washed three times in 0.9% NaCl , lysed by addition of 1 vol. of water, shaken manually with 0.4 vol. of toluene for 5 min. and allowed to stand overnight at 4° . After centrifuging, the haemoglobin layer was removed, globin was precipitated and separated from haemin by addition of acetone containing 1% of HCl according to Anson & Mirsky (1930); the haemin was removed by filtration and globin was washed with acetone and ether.

Isolation of glycine from proteins. Proteins were hydrolysed by heating under reflux with 3000 vol. of 6N- HCl for 48 hr. at 112° . Hydrochloric acid was eliminated and the residue was dissolved in water and evaporated to dryness *in vacuo* six times. Glycine was isolated as dinitrophenylglycine (DNP-glycine), after treatment of the hydrolysate by dinitrofluorobenzene, on a Celite column by a modification by Kruh, Dreyfus, Schapira & Padieu (1957) of Perrone's (1951) method. The purity of DNP-glycine was determined by a two-dimensional paper chromatography according to Levy (1954), by determination of the melting point of the compound and of its mixture with the synthetic compound. The DNP-glycine was dissolved in methanol.

Isolation of free glycine. Trichloroacetic acid was removed with ether and the solution was treated with dinitrofluorobenzene; the DNP-glycine was isolated and its purity determined as described above.

Determination of radioactivity. The proteins were washed

twice with 5% trichloroacetic acid, twice with acetone and once with ether. They were crushed into a fine powder in an agate mortar and spread in layers of 'infinite thickness' on a copper planchet (2.85 cm.^2). The specific activity of DNP-glycine is expressed in counts/min./m-mole. Its concentration was measured with a spectrophotometer at $360\text{ m}\mu$, after dilution with 2% NaHCO_3 ; its radioactivity was determined by drying another portion in a copper planchet in which a thin paper had been fixed with silicone. Determinations were carried out with a standard error of less than $\pm 2\%$; corrections for thickness were made.

RESULTS

The incorporation of glycine into myosin is maximum by 36 hr.; the radioactivity of the myosin remains constant until about the thirtieth day and then decreases to a lower plateau, and the radioactivity of aldolase decreases constantly in animals fed with a diet containing 24% of protein. Table 1 shows a typical result obtained in one series of animals; Fig. 1 shows the results obtained with all the rats. The curve of the specific activity of glycine isolated from myosin (Fig. 2) is similar, and the specific activity of free glycine in muscle decreases exponentially.

Nevertheless, the spread of the experimental points requires other evidence for this interpretation

Table 1. *Radioactivity of myosin and aldolase after injection of [^{14}C]glycine into rats fed with the standard diet (24% of protein)*

One series of rats was used.

Days after injection	Radioactivity (counts/min.)		Ratio: Aldolase/Myosin
	Myosin	Aldolase	
2	41	134	3.3
5	38	112	2.95
11	37	100	2.7
15	47	81	1.7
23	40	70	1.7
26	48	65	1.35
39	20	43	2.15

Fig. 1. *Radioactivity of myosin after injection of [^{14}C]glycine into rats fed with the standard diet.*

of the shape of the radioactivity curve of myosin. The plateau is ascertained by the constant ratio of the radioactivity of myosin to globin between the fifth and the thirtieth days (Fig. 3); this supports an identical behaviour of myosin and globin during this period (later there seems to be a temporary decrease due to the drop of the radioactivity of myosin). The initial decrease of the ratio is due to the lag in the appearance of circulating radioactive red cells: the specific activity of haemoglobin increases for a week, whereas that of myosin reaches its maximum within 2 days after the injection. This decrease in the radioactivity of the myosin is further suggested by the shape of the curve of the ratio of the radioactivity of aldolase or soluble protein of muscle to that of myosin. Fig. 4 shows that the ratio curve is descending first, due to the decrease of the radioactivity of aldolase (Fig. 4A) and of the soluble proteins of muscle (Fig. 4B); after 32 days, the slope of the curve is reversed, which can be explained by the sudden

fall of the radioactivity of the myosin. After 40 days the curve decreases again, since myosin has reached a new plateau. A similar result was obtained in one series in which [^{14}C]valine was used instead of [^{14}C]glycine (Fig. 4C).

The ratio of radioactivity (total protein of myofibrils/myosin), which is 1.21 at the third day, approaches 1 and remains so until the end of the experiment (42 days) (Table 2).

High-protein diet. In rats given a protein-rich diet, the curve has a different shape and does not show a plateau. Table 3 gives the result of one typical experiment. Fig. 5 gives the result for all the animals. The dispersion of the results makes it difficult to draw a curve, but it can be seen that the curve would decrease slowly without displaying any apparent fall. Nevertheless, the ratio of the radioactivity of the soluble protein of muscle to

Fig. 2. Specific activities of glycine in myosin (●) and free glycine (○) of muscle (measured as DNP-glycine) in rats fed with the standard diet, after injection of [^{14}C]glycine.

Fig. 3. Ratio of the radioactivity of myosin to that of globin.

Fig. 4. Ratio of radioactivities of muscle proteins. (A) Aldolase/myosin, after injection of [^{14}C]glycine into rats fed on the diet with 24% of protein (two different series of animals were used). (B) Muscle soluble protein/myosin in the same conditions (two different series of animals were used). (C) Muscle soluble protein/myosin after injection of [^{14}C]valine into rats fed on the diet with 24% of protein. (D) Muscle soluble protein/myosin after injection of [^{14}C]glycine into rats fed on the diet with 45% of protein.

Table 2. Ratio of the radioactivity of total myofibril proteins to that of myosin after injection of [^{14}C]glycine

Rats were fed with a diet containing 24% of protein.

Days after injection	Ratio of radioactivities: myofibril proteins / myosin
3	1.21
9	1.15
14	1.11
24	1.02
28	1.00
35	1.00
42	1.00

Table 3. *Radioactivities of myosin and soluble proteins after injection of [¹⁴C]glycine into rats fed with the high-protein diet (45 % of protein)*

One series of rats was used.

Days after injection	Radioactivity (counts/min.)		Ratio of the radioactivities: soluble proteins: myosin
	Myosin	Soluble proteins of muscle	
4	57	115	2.0
11	63	115	1.85
18	46	69	1.50
25	35	51	1.45
32	45	56	1.25
39	30	48	1.60
48	20	29	1.45
53	25	34	1.35

Fig. 5. Specific activity of glycine in myosin (measured as DNP-glycine) after injection of [¹⁴C]glycine into rats fed with the high-protein diet.

that of myosin (Fig. 4D) presents the same reversal of the slope as that with rats fed with the standard diet.

DISCUSSION

Myosin and life time of myofibrils. The nearly constant radioactivity of myosin (Fig. 1) can be interpreted to mean, as with haemoglobin, that myosin is not in a dynamic equilibrium. In the same way as the behaviour of haemoglobin permits one to determine the life span of the red cells, the behaviour of myosin may permit one to measure the life span of the muscle cell or, more likely, of a sub-unit of the cell.

Recently Leblond & Walker (1956) have discussed the notion of cellular renewal in a large number of cell populations, the cells of the haemopoietic tissues and the red cells being the best-investigated types. Among fixed tissues, the epithelial cells are continuously renewed; on the other hand, the duration of life of hepatic cells has been estimated to be at least 4–5 months (Swick, Koch & Handa, 1956); that of the muscle cells has

not been investigated so far. Their life duration seems to be very long in view of the absence of mitotic activity; the rate of turnover of the various cellular sub-units varies widely and we can interpret our findings in the light of the life span of a subcellular unit likely to be the myofibril. This notion has been considered by several histologists. Whereas the division of the myofibrils has been observed only very rarely, by Adams, Denny-Brown & Pearson (1954), it has been noted that the phenomena of muscle hypertrophy or atrophy was accompanied by variation in the number of the myofibrils. In our experiments the plateau is reached after 2 days and the decline in radioactivity of the myosin takes place between 28 and 35 days. The molecules of myosin are therefore destroyed about 30 days after being synthesized. Since the radioactivity of myosin is very similar to that of the total myofibril proteins, it seems that the curve of the radioactivity of myosin reflects the life span of myofibrils. The decline in over-all radioactivity of the myofibril protein, compared with that of myosin, can be explained by the fact that the myofibril contains a small amount of protein with a fairly rapid turnover, but the bulk of its protein is composed of myosin and perhaps other proteins with an apparent metabolic inertness and the same radioactivity as myosin. These results permit us to ascribe to the myofibrils of the rat a life span of 30 days (unpublished experiments have shown a life duration for mice myofibrils of 20 days). The values given are only average values for the whole animals, since the incorporation of the amino acid may vary with the site of the muscle, as has been shown previously with [¹⁵N]-glycine by Schapira, Coursaget, Dreyfus & Schapira (1953). Variation of incorporation with the site has also been shown in epidermal and intestinal cells by Hooper (1956). However, our results demonstrate the possibility of determining the life span of a subcellular unit by biochemical methods.

Myosin and dynamic equilibrium. The experiments performed with rats fed with a diet containing 24 % of protein led us to conclude that myosin is metabolically inert or almost inert. However, this inertness may only be apparent since free glycine of muscle remains significantly radioactive for several weeks, as we have shown previously (Schapira, Dreyfus, Kruh, Labie & Padieu, 1958). This radioactive precursor should tend to increase the specific activity of myosin, even after the first days: if myosin was completely inert for 30 days, the specific activity of free glycine would be enough to increase the over-all activity of myosin, since at that time only non-radioactive myosin would be destroyed. Since we observed a plateau and no increase, it is probable that either some molecules of radioactive myosin are destroyed

during the life of the myofibril (compensating for the new molecules of radioactive myosin which are being synthesized) or that there is some turnover of the myosin molecule which decreases progressively its radioactivity. The plateau would, in this case, represent the net result of the two mechanisms. To demonstrate this we decided to feed rats with a diet enriched in protein, in order to dilute as fast as possible the free radioactive glycine.

Steinbock & Tarver (1954) have shown that a protein-rich diet apparently accelerated the renewal of proteins, a possible consequence of the decrease in re-utilization of the radioactive amino acid. In the experiments with a protein-rich diet we observed a disappearance of the plateau and the radioactivity progressively decreased, with a large dispersion of the points. It appears, under these conditions, that a molecular renewal of myosin is demonstrated. It is impossible to demonstrate from the curves the life duration of myofibrils: our conclusion is, however, supported by the fact that by about the thirtieth day, the slope of the curve of radioactivities (water-soluble protein/myosin) is reversed (Fig. 4D).

We can therefore come to the following conclusions: the myofibrils display a definite life span of about 30 days; the myosin is in a state of relative inertness and its metabolic behaviour can be compared with that of haemoglobin; the inertness, however, is not complete; if the radioactive free glycine is diluted by the use of a high-protein diet, the myosin molecule appears to have a slow but definite molecular turnover.

SUMMARY

1. After the injection of radioactive glycine into groups of rats on a diet containing 24 % of protein, myosin was isolated at various times.
2. The specific activity of the myosin (and of the glycine of the myosin) remained constant for about 30 days and then decreased.
3. The ratio of the specific activity of the myosin to that of erythrocyte globin was constant between about 5 and 30 days after injection.
4. On this basis the life span of the myofibril is considered to be 30 days on average.
5. When the animals received a diet containing 45 % of protein the radioactivity of myosin pro-

gressively decreased, showing that the protein undergoes turnover.

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REFERENCES

- Adams, R. D., Denny-Brown, D. & Pearson, C. M. (1954). *Diseases of Muscle*. New York: P. B. Hoeber.
- Anson, M. L. & Mirsky, A. E. (1930). *J. gen. Physiol.* **17**, 369.
- Bidinost, L. E. (1951). *J. biol. Chem.* **190**, 423.
- Dreyfus, J. C., Kruh, J. & Schapira, G. (1957). *Comm. Conf. int. Radioisotopes Rech. sci., Paris*. UNESCO NS/RIC No. 89.
- Dreyfus, J. C., Schapira, G. & Kruh, J. (1956). *C.R. Soc. Biol., Paris*, **150**, 1145.
- Hooper, C. E. (1956). *J. Histochem. Cytochem.* **4**, 531.
- Kruh, J., Dreyfus, J. C., Schapira, G. & Padieu, P. (1957). *J. biol. Chem.* **228**, 113.
- Leblond, C. P. & Walker, B. E. (1956). *Physiol. Rev.* **36**, 255.
- Levy, A. L. (1954). *Nature, Lond.*, **174**, 126.
- Miller, L. L., Bly, D. G., Watson, M. L. & Bayle, W. F. (1951). *J. exp. Med.* **94**, 431.
- Neuberger, A. (1952). *Brit. med. Bull.* **8**, 210.
- Neuberger, A., Perrone, J. C. & Slack, H. G. B. (1951). *Biochem. J.* **49**, 199.
- Perrone, J. C. (1951). *Nature, Lond.*, **167**, 513.
- Perry, S. V. (1953). *Biochem. J.* **55**, 114.
- Robertson, W. van B. (1952). *J. biol. Chem.* **197**, 495.
- Schapira, G., Coursaget, J., Dreyfus, J. C. & Schapira, F. (1953). *Bull. Soc. Chim. biol., Paris*, **35**, 1309.
- Schapira, G., Dreyfus, J. C., Kruh, J., Labie, D. & Padieu, P. (1958). *2ième Conf. int. Nations Unies Energie Atomique, Genève*. A.I/P No. 1229.
- Shemin, D. & Rittenberg, D. (1944). *J. biol. Chem.* **153**, 401.
- Shemin, D. & Rittenberg, D. (1945). *J. biol. Chem.* **159**, 567.
- Steinbock, H. J. & Tarver, H. (1954). *J. biol. Chem.* **209**, 127.
- Swick, R. W., Koch, A. L. & Handa, D. T. (1956). *Arch. Biochem. Biophys.* **63**, 226.
- Szent-Gyorgyi, A. G. (1951). *J. biol. Chem.* **192**, 361.
- Taylor, J. F., Green, A. A. & Cori, G. T. (1948). *J. biol. Chem.* **173**, 591.
- Thompson, R. C. & Ballou, J. E. (1956). *J. biol. Chem.* **223**, 795.
- Warburg, O. & Christian, W. (1943). *Biochem. Z.* **314**, 19.