the animals and not by scurvy was very much less marked than that observed in scorbutic guinea pigs.

8. It is assumed that the fall of the phosphatase in the zones of provisional calcification of scorbutic guinea pigs may be due to a disturbance in the function of the osteoblasts caused by scorbutic lesions. The evidence so far obtained, however, makes it probable that the early diminution in the serum phosphatase is not due to a disturbance in the production of the enzyme, as in the case of the bones, but rather to its removal from the circulation to fulfil some protective function. The evidence also does not exclude the possibility that the serum phosphatase of healthy animals is of vascular origin.

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Chemical Studies of Peripheral Nerve During Wallerian Degeneration

2. LIPIDS AFTER NERVE CRUSH (AXONOTMESIS)

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In a previous paper the changes that occur in the lipids of a peripheral nerve during Wallerian degeneration were described (Johnson, McNabb & Rossiter, 1949*b*). The sciatic nerve was sectioned in a number of cats and the peripheral segment was allowed to degenerate. Special precautions were taken to minimize the possibility of re-innervation. In the experiments now to be reported the nerve was gently crushed, a procedure that causes the distal segment to undergo a series of changes similar to those of Wallerian degeneration. After a short latent period the degenerating nerve is then re-innervated by axon tips from the intact central stump. This type of lesion has been called axonotmesis by Seddon (1943) and must be distinguished from neurotmesis, the type of lesion previously investigated.

The changes in the nerve are essentially those of Wallerian degeneration, upon which are superimposed those of regeneration. Previous studies in this field have been for the most part either histological or physiological. Excellent reviews are those of Howell & Huber (1892), Kirk & Lewis (1917), Ramon y Cajal (1928), Spielmeyer (1929), Nageotte (1932), Weiss (1944) and Young (1942, 1945, 1948). In few instances have the techniques of chemistry been employed. The most notable exceptions are the studies of Mott & Halliburton (1901), who measured the phosphorus content of regenerating cat nerves; and Abercrombie & Johnson (1947), who reported changes in the distribution of nitrogen, including collagen nitrogen, in regenerating rabbit nerves.

METHODS

The right sciatic nerve of thirty-eight adult cats was gently crushed at the level of the greater trochanter of the femur with a crushing clamp. This procedure interrupts the axons and the myelin sheaths, leaving the connective-tissue sheaths intact. The operation was performed under nembutal anaesthesia with full aseptic precautions. No attempt was made to control the age, sex, or weight of the animals. After periods of time varying from 16 to 144 days the animals were killed and the segment of the nerve distal to the site of the crush was removed. At the same time, a similar length

of left sciatic nerve was removed to serve as a control. Each nerve was cleaned of adherent fatty and epineurial connective tissue, weighed, and the lipids extracted and estimated as described previously (Johnson *et al.* 1949*b*). The concentration of cerebroside (glycosphingoside), total and free cholesterol, total phospholipin, monoaminophospholipin (phosphoglyceride), lecithin (phosphatidyl choline), and neutral fat was determined in each nerve, and from these figures the concentration of ester cholesterol, sphingomyelin (phosphosphingoside), kephalin, myelin lipid and total lipid was calculated. Analyses were done in duplicate. Details of the analytical procedures, together with a discussion of their specificity and accuracy, are given in the previous paper by Johnson *et al.* (1949*b*).

The results for the regenerating nerves have been expressed in terms of the fresh weight of a similar length of the intact nerve from the opposite side. This is equivalent to expressing the results in terms of the fresh weight of the nerve before it had been crushed, i.e. at zero time.

Except for neutral fat, the values given for the control nerves represent the pooled means for all the animals of this series, together with those of the degeneration series previously published. The figures are thus derived from observations made on sixty-eight different cats and give a good indication of the concentration of the lipids in normal cat nerve.

RESULTS

All the animals suffered a partial paralysis of the right hind limb, but despite this they were able to move about with little apparent inconvenience. An animal kept alive for sufficient time would usually develop a trophic ulcer of the right foot. By 60 days, however, such an animal was able to walk on the toes of the affected leg, and the ulcer had begun to heal. After 80 days there was apparently a full return of function to the whole leg, and the ulcer had completely healed. The muscles of the affected limb were greatly wasted.

Table 1 gives the mean and the standard error of the mean for the concentration of each of the lipids in the control nerves and in the experimental nerves 48 and 144 days after the operation. The table also gives the value of phosphorus obtained for each lipid in testing the significance of the difference between the means of the values for the control and experimental nerves.

By 48 days the concentration of each of the lipids in the experimental nerves, with the exception of ester cholesterol and neutral fat, was considerably less than that of the corresponding lipid in the control nerves. By 144 days the concentration of each of these lipids had increased, but it was still significantly less than that in the control nerves. By this time ester cholesterol and neutral fat were the only lipids that were present in a concentration similar to that in the control nerves.

Table 1. *Lipids of cat sciatic nerve after nerve crush*

(Number of animals is stated in parentheses under each result. Results as mg./100 mg. wet weight of control nerve.)

	Control	No. of days after crush: 48 days		144 days	
	Mean S.E.M.	Mean S.E.M.	P	Mean S.E.M.	P
Cerebroside (*a*)	2·12 ± 0·08 (66)	0·57 ± 0·15 (4)	<0·01	0·78 ± 0·16 (5)	<0·01
Total cholesterol (*b*)	3·29 ± 0·03 (68)	1·70 ± 0·17 (4)	<0·01	1·26 ± 0·10 (5)	<0·01
Free cholesterol (*c*)	3·26 ± 0·03 (68)	0·92 ± 0·08 (4)	<0·01	1·22 ± 0·10 (5)	<0·01
Ester cholesterol ($b-c$)	0·03 ± 0·01 (68)	0·78 ± 0·10 (4)	<0·01	0·05 ± 0·01 (5)	>0·4
Total phospholipin (*d*)	6·03 ± 0·05 (66)	1·63 ± 0·09 (4)	<0·01	2·80 ± 0·13 (4)	<0·01
Monoaminophospholipin (*e*)	3·71 ± 0·07 (66)	0·98 ± 0·09 (4)	<0·01	1·45 ± 0·07 (4)	<0·01
Lecithin (*f*)	0·78 ± 0·02 (66)	0·49 ± 0·03 (4)	<0·01	0·45 ± 0·03 (4)	<0·02
Sphingomyelin ($d-e$)	2·32 ± 0·07 (66)	0·65 ± 0·12 (4)	<0·01	1·35 ± 0·05 (4)	<0·01
Kephalin ($e-f$)	2·93 ± 0·07 (66)	0·47 ± 0·16 (4)	<0·01	1·00 ± 0·06 (4)	<0·01
Myelin lipid ($a+c+d-e$)	7·69 ± 0·13 (64)	2·17 ± 0·24 (4)	<0·01	3·42 ± 0·29 (4)	<0·01
Neutral fat (*g*)	8·91 ± 0·35 (36)	7·84 ± 1·26 (3)	>0·4	10·33 ± 0·27 (5)	>0·1
Total lipid ($a+b+d+g$)	19·59 ± 0·32 (51)	11·60 ± 1·74 (3)	<0·01	15·40 ± 0·53 (4)	<0·01

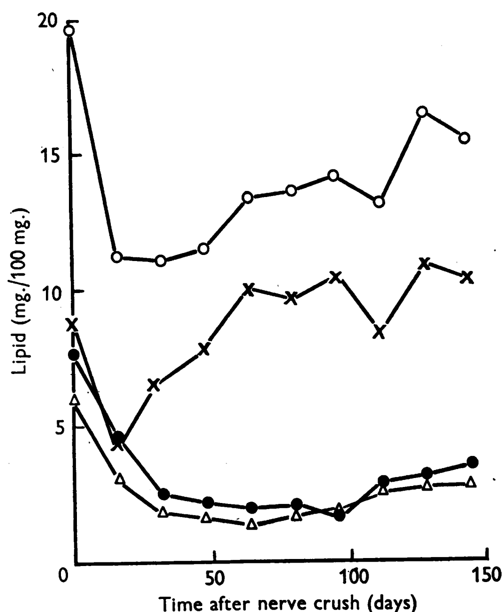

Fig. 1. Lipids of cat sciatic nerve after nerve crush (axonotmesis). ○—○, total lipid; ×—×, neutral fat; ●—●, myelin lipid; △—△, total phospholipin. Concentration of lipids is expressed as mg./100 mg. wet weight of control nerve. Each point represents the mean of figures for four or more animals.

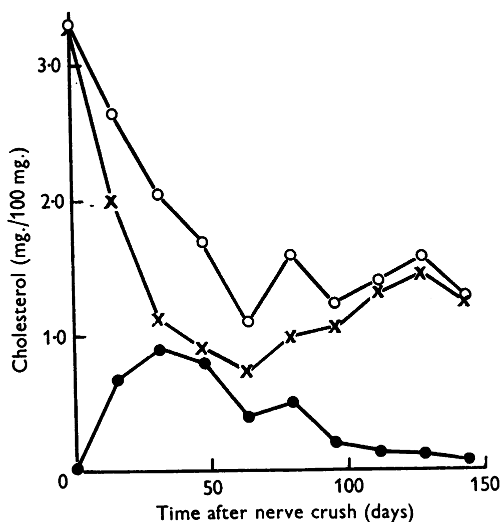

Fig. 2. Cholesterol of cat sciatic nerve after nerve crush (axonotmesis). ○—○, total cholesterol; ×—×, free cholesterol; ●—●, ester cholesterol. Concentration of cholesterol is expressed as mg./100 mg. wet weight of control nerve. Each point represents the mean of figures for four or more animals.

Fig. 1 shows that the concentration of total lipid decreased rapidly during the first 16 days, remained relatively constant during the period 16–48 days, and then increased slowly. In contrast, the concentration of myelin lipid and phospholipin decreased steadily during the first 64 days, changed little during the period 64–96 days, and then commenced to increase. Much of the increase in the concentration of total lipid 48 days after the operation was the result of an increase in the concentration of neutral fat.

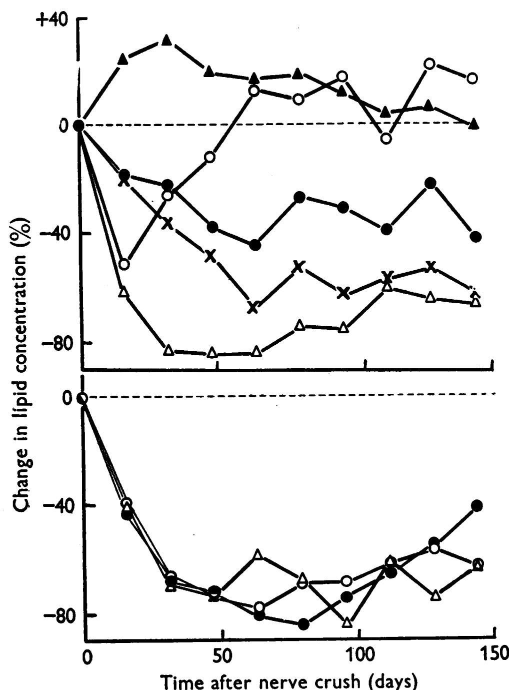

Fig. 3. Percentage change in the concentration of lipids in cat sciatic nerve after nerve crush (axonotmesis). Above, non-myelin lipids; ○—○, neutral fat; ●—● lecithin; ×—×, total cholesterol; △—△, kephalin; ▲—▲, wet weight. Below, myelin lipids; ○—○, free cholesterol; △—△, cerebroside; ●—●, sphingomyelin. Each point represents the mean of figures for four or more animals.

Fig. 2 shows the changes in cholesterol. The concentration of free cholesterol, which accounted for almost all of the cholesterol in the control nerves, decreased rapidly during the first 32 days, and by 64 days had commenced to increase. The concentration of ester cholesterol, absent from control nerves, increased rapidly, reaching a maximum in 32 days. The concentration of ester cholesterol then gradually decreased, until by 144 days the figures were not significantly different from those for the control nerves.

Fig. 3 (bottom) shows that the percentage change in the concentration of each of the myelin lipids, i.e.

free cholesterol, cerebroside and sphingomyelin, was similar throughout the whole 144-day period. On the other hand, the percentage change in the concentration of the non-myelin lipids was different (Fig. 3, top). Total cholesterol and lecithin decreased less rapidly than the myelin lipid, while kephalin decreased more rapidly.

Fig. 3 (top) also shows the percentage change in the wet weight of the crushed nerves. The wet weight rapidly increased after the operation, but the animal to animal variation was such that this increase was significant statistically ($P < 0{\cdot}05$) only at 32 days.

DISCUSSION

When a peripheral nerve is crushed the segment distal to the site of the crush degenerates. The axon disintegrates and disappears, while the myelin sheath at first fragments and ultimately is destroyed. The Schwann cells proliferate and the nerve sheaths thicken to become Schwann tubes, which are able to receive a growing axon tip from the intact central stump. The degeneration is, in a sense, a preparation for regeneration. Most recent studies on nerve regeneration have confirmed the early views of Howell & Huber (1892) and Kirk & Lewis (1917) that myelination spreads down the nerve in a peripheral direction. The myelin sheaths of the fibres of a regenerated nerve appear similar to those of a normal nerve, and it is generally assumed that regenerated 'myelin' has the same chemical composition as normal 'myelin'. That the myelin sheaths are not similar in all respects is shown by the interesting finding of Vizoso & Young (1948) that, whereas in normal nerves the distance between the nodes of Ranvier is proportional to the diameter of the nerve fibre, in regenerated nerves the internodal distance tends to be constant.

Previous studies from this laboratory have indicated that free cholesterol and the two lipids that contain the base sphingosine,* cerebroside and sphingomyelin, are the principal lipid components of the myelin sheath of a mammalian nerve fibre (Johnson, McNabb & Rossiter, 1948*a*, *b*; 1949*a*, *b*). We have called these lipids the 'myelin lipids' while Brante (1949), who has made a similar study, refers to them as being 'sheath typical'. It in no way follows that these three are the only lipids contained in the myelin sheath, but they are the lipids present in the greatest concentration and those that distinguish the myelin sheath from other parts of the nervous system. The myelin sheath may well contain some lecithin, but our analyses show that much lecithin is present in other non-myelin structures of the nervous system. Since, as Folch (1949) has shown, the kephalin of the nervous system is a mixture of at least three individual lipids (phosphatidyl serine, phosphatidyl ethanolamine and brain diphosphoinositide), the exclusion of kephalin from the myelin lipids does not necessarily exclude any of the individual constituents of kephalin. Brante (1949) has published some data supporting the view that phosphatidyl serine is a myelin lipid, but the position of phosphatidyl ethanolamine and diphosphoinositide is not so clear. More data are required on these points.

In a crushed peripheral nerve one would expect to find a decrease in the concentration of myelin lipid during the stage of degeneration, followed by an increase as the regenerating fibres become myelinated and the myelination spreads down each fibre. For the first 32 days after the operation the changes in the concentration of the lipids were similar to those reported previously for sectioned nerves (Johnson *et al.* 1949*b*). After 32 days, however, differences became apparent. Whereas the concentration of myelin lipid in the sectioned nerves continued to fall during the period 32–96 days, the concentration of myelin lipid in the crushed nerves remained fairly constant during this period, and after 96 days it gradually increased. The individual myelin lipids, cerebroside, free cholesterol and sphingomyelin, followed a course similar to that of total myelin lipid.

There can be little doubt that the gradual increase in the concentration of myelin and other lipids after 96 days represents the formation of new myelin sheaths and other lipid-containing structures in the regenerating nerve. The analyses were done on the whole nerve, and the lipids measured represent not only those of the myelin sheath, but also those of the axon, Schwann cells, endoneurium, and perineurial connective tissue. Since the greater part of the non-triglyceride lipid of a peripheral nerve is in the myelin sheath, it is reasonable to interpret these changes in terms of degeneration and regeneration of 'myelin', but the changes in other nerve structures must not be forgotten.

Of interest is the appearance of cholesterol ester in both sectional and crushed nerves. Cholesterol ester is not usually present in either the central or the peripheral nervous system, but it appears during the demyelination process. After 96 days the concentration of ester cholesterol in re-innervated nerves was less than that previously found for nerves in which there was no re-innervation (Johnson *et al.* 1949*b*). This would suggest that some of the cholesterol ester hydrolyses to form free cholesterol, which is incorporated in the new myelin sheaths (Fig. 2).

An outstanding feature of our results is that by 144 days the concentration of myelin lipid in the crushed nerves was still much less than that in the

* Carter, Haines, Ledyard & Norris (1947) have suggested that the sphingosine-containing lipids (e.g. cerebroside and sphingomyelin) be classified as sphingolipins, a term accepted by Folch-Pi & Sperry (1948).

controls. Microscopic examination confirmed that the nerves had degenerated and subsequently had become re-innervated. By 80 days the myelination had spread throughout the whole nerve, and function appeared normal. No attempt was made to measure the rate of regeneration as was done by Gutmann, Guttmann, Medawar & Young (1942). Gutmann & Sanders (1943) and Sanders (1948) found that the fibre pattern of a nerve 144 days after crushing is far from normal, and so also is its excitability and the rate at which it can conduct an impulse (Berry, Grundfest & Hinsey, 1944; Sanders & Whitteridge, 1946; Erlanger & Schoepfle, 1946). Apparently, regenerated nerves are in many ways different from normal nerves. In this connexion it is interesting to note that Abercrombie & Johnson (1947) found that in crushed sciatic nerves of the rabbit the concentration of both collagen and protein nitrogen 100 days after the operation was much greater than that in control nerves.

As far as we are aware, no comprehensive study has previously been made of the chemical changes in regenerating nerves. Our findings on the increase in wet weight agree with those of Mott & Halliburton (1901) and Abercrombie & Johnson (1947). Mott & Halliburton (1901) also observed that the phosphorus had completely disappeared from sectioned sciatic nerves of the cat by 29 days, but that, in two animals in which there was physiological evidence of regeneration, the phosphorus concentration in the nerves 100 days after the operation was almost 80 % of that in the control nerves. It should be noted that Mott & Halliburton (1901) measured total phosphorus, whereas in our experiments lipid phosphorus only was determined.

SUMMARY

1. The concentrations of cerebroside, total and free cholesterol, total phospholipin, monoaminophospholipin, lecithin and neutral fat were determined in cat sciatic nerves at intervals of time from 16 to 144 days after nerve crush (axonotmesis). From these figures the concentrations of ester cholesterol, sphingomyelin, kephalin, myelin lipid and total lipid were calculated.

2. The wet weight of the nerve increased after the operation, reaching a maximum in 32 days and returning to normal by 144 days.

3. The total lipid content of the nerve decreased during the first 16 days, remained constant during the period 16–48 days and then increased gradually. Even after 144 days the concentration of total lipid was less than that of the control nerves.

4. Neutral fat decreased rapidly during the first 16 days, but had returned to normal by 48 days.

5. The myelin lipids (cerebroside, free cholesterol and sphingomyelin) decreased during the first 32 days, remained constant during the period 32–96 days and then increased gradually. By 144 days the concentration of myelin lipid was 44 % of the value for the control nerves.

6. Both total and free cholesterol decreased during the first 32 days, free cholesterol more rapidly than total, for cholesterol ester, absent in control nerves, appeared during this period. The free cholesterol followed a course similar to that of myelin lipid while the cholesterol ester disappeared, none remaining at 144 days.

7. Total phospholipin decreased during the first 32 days, remained fairly constant during the period 32–96 days and then increased gradually. Sphingomyelin followed a similar course, but kephalin decreased more rapidly and lecithin more slowly. Lecithin did not return to the nerve, the concentration at 144 days being similar to that at 64 days.

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The Interaction of Serum Albumins with Calcium

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Pribram in 1871 postulated that some part of the serum calcium was bound to the serum proteins. Rona & Takahashi (1911) produced experimental evidence justifying Pribram's suggestion. Numerous authors (Loeb, 1926; Marrack & Thacker, 1926; Greenberg & Greenberg, 1931; McLean & Hastings, 1935; Weir & Hastings, 1936; Drinker, Green & Hastings, 1939; Ludewig, Chanutin & Masket, 1942) have published papers on qualitative and quantitative studies of the distribution of calcium in human serum, and the extent to which it may combine with human and other proteins.

McLean & Hastings (1935) gave tables of pK values for calcium proteinate based on a hypothetical mass law relationship

$$\frac{[\mathrm{Ca}^{++}]\,[\mathrm{Protein}^{--}]}{[\mathrm{Ca\ protein}]}=\mathrm{K},$$

and in the same paper developed a nomogram based on this mass law to explain the relationships in human serum. Weir & Hastings (1936) produced further data to support this relationship, but revised the pK values. Ludewig *et al.* (1942) re-examined the relationship of calcium to protein in human serum, using the ultracentrifuge to study the equilibrium. They concluded that the system was too complex for the simple mass law to be applied.

Recent developments in the technique of low-temperature fractionation of proteins (Pillemer & Hutchinson, 1945; Cohn, Strong, Hughes, Mulford, Ashworth, Melin & Taylor, 1946; Kekwick & MacKay, 1949) offered fresh opportunity for the examination of the relationships of calcium to the individual serum proteins. Of these, albumin seemed the most satisfactorily characterized for careful study. In the present investigation we started with the object of discovering whether the calcium-binding capacities of albumins of different species are the same, and whether albumins from the same species, separated and purified by different techniques, have the same calcium-binding capacity.

EXPERIMENTAL

The albumins examined had been prepared by the methods of Cohn *et al.* (1946), Pillemer & Hutchinson (1945) and Kekwick & MacKay (1949). The purity of the specimens was judged by electrophoretic analysis carried out in the 11 cm. analytical cell in phosphate buffer, ionic strength 0·2, at pH 8·0, using the moving-boundary technique of Tiselius (1939).

The Ca distribution was studied by dialysis. These dialyses were carried out at 2° in a two-chambered system separated by a semi-permeable membrane. The time taken to establish equilibrium was measured by controls run in parallel. Though equilibrium in the majority of cases was established in 72 hr. all specimens were left for 6 days before analysis of the ionic distribution between the two chambers was carried out. During this period the samples were stirred frequently to help to establish equilibrium. The ratio of the volume of fluid in the protein-free compartment to that in the protein-containing compartment was 15 : 1.

Determinations of pH were carried out on all samples at the completion of each experiment, using a glass electrode and the Cambridge potentiometer.

Ca determinations on the fluid in each compartment were made by a modification of the method of Trevan & Bainbridge (1926). Fig. 1 shows the percentage deviation of Ca estimated by our method, from Ca concentrations estimated gravimetrically on large samples.

Chloride determinations were carried out on the fluid of both compartments by the method of Van Slyke (1923).

The protein concentrations in the inner compartment were determined at the completion of each equilibrium by N determination using the standard micro-Kjeldahl technique. In each instance the fluid in the outer compartment was examined, and any experiment in which transference of N-containing materials could be demonstrated was rejected. The albumins had been dialysed against 0·15 M-NaCl prior