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Chemical Studies of Peripheral Nerve During Wallerian Degeneration

5. β-GLUCURONIDASE

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In an endeavour to outline more fully the chemical changes that take place in a peripheral nerve after nerve section or nerve crush, measurements have been made of the activity of the enzyme β-glucuronidase.

Previous papers in this series have described the changes that occur in the concentration of lipids (Johnson, McNabb & Rossiter, 1949; Burt, McNabb & Rossiter, 1950) and protein-bound phosphorus compounds (Logan, Mannell & Rossiter, 1952) and in the activity of phosphatases (Hollinger, Rossiter & Upmalis, 1952) in peripheral nerve after either nerve section or nerve crush.

METHODS

The sciatic nerve on one side of each of a series of fifty-six cats was either sectioned or crushed high in the thigh. Details of the operations have already been described (Johnson *et al.* 1949; Burt *et al.* 1950). No attempt was made to control the age, sex or weight of the animals. After intervals of time varying from 1 to 600 days the animals were killed and the distal degenerating or regenerating segment of the nerve was removed. At the same time a similar length of nerve of the opposite side was taken to serve as a control. The two pieces of nerve were homogenized in 10 ml. isotonic saline as described by Hollinger *et al.* (1952).

Determination of β-glucuronidase activity. The β-glucuronidase activity of portions of the homogenate was determined by the method of Fishman, Springer & Brunetti (1948) using the biosynthetic substrate, phenolphthalein mono-β-glucuronide, prepared as described by these workers. The reaction mixture contained 0·1 ml. homogenate (diluted by a factor of 3–5 for nerves of high activity), 1 ml. 0·1 M-acetate buffer, pH 4·5, and 0·1 ml. 0·01 M-phenolphthalein mono-β-glucuronide. This mixture was incubated at 37° for 4 hr., at the end of which time the reaction was stopped by the addition of 1 ml. 20% (w/v) trichloroacetic acid. A control tube containing enzyme preparation and buffer, but no substrate, was incubated for the same length of time. Substrate was added to this tube immediately before the addition of the trichloroacetic acid. The determination was completed as described by Fishman *et al.* (1948), the test and control being read against a blank containing buffer and trichloroacetic acid in a Coleman universal spectrophotometer at 540 mμ. A standard containing 20 μg. phenolphthalein/tube was read with each set of determinations. The test was always run in duplicate.

It will be noted that the determination was done on a portion of the whole homogenate and not, as is sometimes customary (Kerr & Levvy, 1951; Walker & Levvy, 1951), on a tissue extract. Under our conditions the rate of production of free phenolphthalein was constant throughout the 4 hr. incubation period. Each tube contained no more than 5 mg. wet tissue in a total volume of 1·2 ml. and for some of the degenerating nerves this was reduced to as little as 1 mg. In this range, the enzyme activity was not a function of the concentration of the homogenate, nor was it increased by the addition of detergents.

Recording of results. The results are reported in terms of phenolphthalein (in μg.) liberated/hr./mg. wet wt. of nerve. These units are equivalent to the glucuronidase units of Fishman *et al.* (1948). Because of the great increase in the wet weight of the degenerating and regenerating nerves, we have, as in the paper by Hollinger *et al.* (1952), expressed the results in terms of the wet weight of a similar length of the control nerve of the opposite side. This is equivalent to expressing the results in terms of the fresh weight of the nerve before it had been sectioned or crushed, i.e. at zero time. In six animals not subjected to any operative procedure the activity of the right nerve did not differ significantly from that of the left when expressed on this basis.

RESULTS

Table 1 shows that 16 days after the nerve was either sectioned or crushed the mean activity of the β-glucuronidase had increased by a factor of 30–40. The mean increase for the crushed nerves was not significantly different from that for the sectioned nerves. Fig. 1 shows the time course of the mean percentage change in the activity of the enzyme after nerve section. On the same figure is shown the much smaller increase in the activity of acid phosphomonoesterase previously reported by Hollinger *et al.* (1952). The similar changes after nerve crush are shown in Fig. 2. At no time up to 96 days did the values for the crushed nerves significantly differ from those for the sectioned nerves. At both 170–180 and 600 days the β-glucuronidase activity of the crushed nerves was still significantly greater than that of the control nerves. At 600 days the mean increase was 30% ± 4.

Table 1. *β-Glucuronidase activity of the sciatic nerve of the cat 16 days after either nerve section or nerve crush*

(Substrate, phenolphthalein mono-β-glucuronide. pH 4·5. Incubation time, 4 hr. Temp. 37°.)

Enzyme activity (μg. phenolphthalein/hr./mg. wet wt.)			
Normal	After nerve section (16 days)	Increase	Percentage increase
0·45	9·45	9·00	2000
0·43	11·15	10·72	2470
0·41	19·45	19·04	4650
0·37	10·00	9·63	2610
0·35	15·30	14·95	4270
0·26	11·60	11·34	4350
		Mean (±S.E.M.) = **3400** ± 480	

Enzyme activity (μg. phenolphthalein/hr./mg. wet wt.)			
Normal	After nerve crush (16 days)	Increase	Percentage increase
0·31	7·10	6·79	2180
0·25	13·75	13·50	5400
0·30	11·95	11·65	3880
		Mean (±S.E.M.) = **3820** ± 890	

Logan *et al.* (1952) described an increase in the concentration of both deoxypentosenucleic acid and pentosenucleic acid after nerve section. In Table 2 the mean activity of β-glucuronidase in normal nerves and that in degenerating nerves 16 days after nerve section are compared with the activities of acid phosphomonoesterase (Hollinger *et al.* 1952) and the concentrations of deoxypentosenucleic acid (Logan *et al.* 1952) at corresponding time intervals. It can be seen that the increase in the activity of acid phosphomonoesterase was of the same order as the increase in the concentration of deoxypentosenucleic acid, but that the increase in the activity of β-glucuronidase was considerably greater.

If the mean quantity of deoxypentosenucleic acid per diploid cell is of the same order for any given species (evidence summarized by Davidson, Leslie & White, 1951) the amount of deoxypentosenucleic acid gives an estimate of the total number of cells throughout the entire length of the nerve. Table 2 shows that, whereas the mean acid phosphomonoesterase activity per μg. deoxypentosenucleic acid P had increased only slightly (48%) at the end of 16 days, the mean β-glucuronidase activity per μg. deoxypentosenucleic acid P, i.e. the mean activity per cell, had increased by over 1100%.

DISCUSSION

As far as we are aware, this is the first report on the β-glucuronidase activity of peripheral nerve. The value for cat sciatic nerve (0·35 μg. phenolphthalein/hr./mg. wet weight) corresponds to 0·35 Fishman units/mg. This figure is less than that reported by Talalay, Fishman & Huggins (1946) for most tissues, but the figure of 12·8 Fishman units/mg. for degenerating nerve is of the same order as that found for rat liver by these workers and is greater than the figures they report for all other rat tissues with the exception of spleen. The activity in the sciatic nerve of the cat is eight times greater than that reported by McNabb (1951) for the brain of the dog.

The great increase in the activity of β-glucuronidase during Wallerian degeneration is of interest, since it has been suggested that β-glucuronidase activity may be associated with cellular proliferation generally (Levvy, Kerr & Campbell, 1948; Kerr, Campbell & Levvy, 1949, 1950). It is also noteworthy that Fishman (1947) showed that the administration of oestrogen to castrated female mice caused an increase in the activity of β-glucuronidase in the uterus. Fishman and his colleagues have also demonstrated a high β-glucuronidase activity in

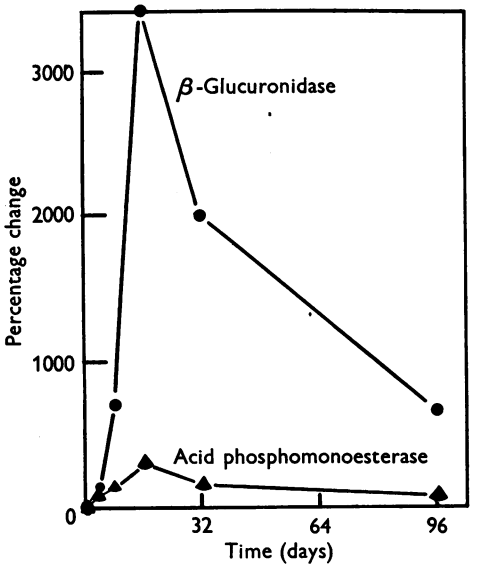

Fig. 1. Mean percentage change in the activity of β-glucuronidase (●-●-●) and acid phosphomonoesterase (▲-▲-▲) in the sciatic nerve of the cat after nerve section. Each point is the mean of three or more determinations.

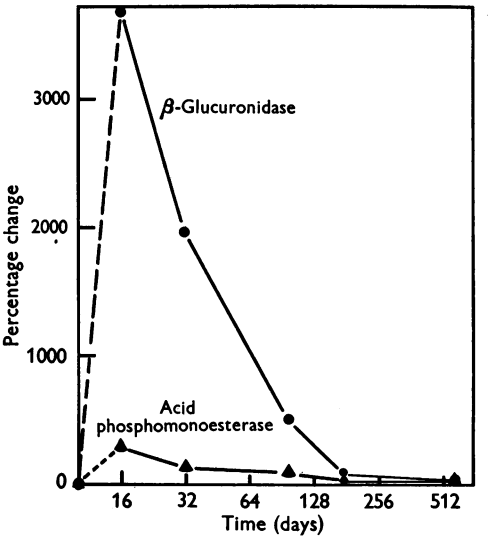

Fig. 2. Mean percentage change in the activity of β-glucuronidase (●-●-●) and acid phosphomonoesterase (▲-▲-▲) in the sciatic nerve of the cat after nerve crush. Each point is the mean of three or more determinations.

Table 2. *β-Glucuronidase activity, acid phosphomonoesterase activity and deoxypentosenucleic acid concentration in the sciatic nerve of the cat 16 days after nerve section*

(Mean ± S.E.M. Figures in parentheses indicate the number of animals in each group.)

	Normal	After nerve section (16 days)	Percentage increase
β-Glucuronidase (μg. phenolphthalein/hr./mg. wet wt.)	0·35 ± 0·013 (56)	12·8 ± 1·60 (6)	3560
Acid phosphomonoesterase (μg. P/hr./mg. wet wt.)	0·37 ± 0·013 (56)	1·64 ± 0·188 (7)	344
Deoxypentosenucleic acid (μg. P/100 mg. wet wt.)	4·8 ± 0·1 (52)	14·4 ± 0·9 (6)	200
β-Glucuronidase (μg. phenolphthalein/hr./μg. deoxypentosenucleic acid P)	7·3	88·9	1120
Acid phosphomonoesterase (μg. P/hr./μg. deoxypentosenucleic acid P)	7·7	11·4	48

certain tumours (Fishman & Anlyan, 1947; Fishman Anlyan & Gordon, 1947). These results were later confirmed and extended by Odell & Burt (1949). In addition, Bernard & Odell (1950) reported changes in the uterus and ovary of the rat during pregnancy and Odell & Fishman (1950) described cyclic changes in the β-glucuronidase activity of specimens of endometrium obtained at biopsy during the different phases of the menstrual cycle in the human female.

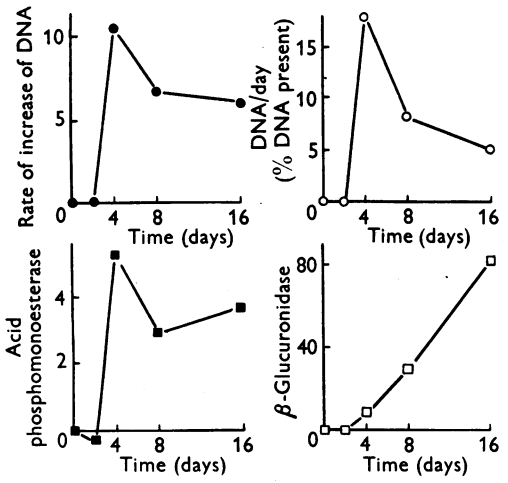

Fig. 3. Comparison of rate of increase of deoxypentosenucleic acid with increase of enzyme activity in the sciatic nerve of the cat after nerve section. Above, left: mean rate of increase of deoxypentosenucleic acid expressed as μg. P/day/g. wet wt. of nerve (●–●–●). Above, right: mean daily increase of deoxypentosenucleic acid expressed as a percentage of deoxypentosenucleic acid already present (○–○–○). Below, left: mean increase in acid phosphomonoesterase activity expressed as μg. P/hr./μg. deoxypentosenucleic acid P (■–■–■). Below, right: mean increase in β-glucuronidase activity expressed as μg. phenolphthalein/hr./μg. deoxypentosenucleic acid P (□–□–□).

The finding that the activity of β-glucuronidase per unit deoxypentosenucleic acid P increases in degenerating nerves, together with the observations mentioned in the preceding paragraph, could all be brought together in the unified hypothesis of Levvy and his associates that the β-glucuronidase activity increases in conditions associated with a cellular proliferation. In degenerating nerve there is certainly a cellular proliferation and there is also a great increase in β-glucuronidase activity. However, the time relations are difficult to reconcile with such an hypothesis. Fig. 3 shows an attempt to determine the time of maximum cellular proliferation. Two chemical estimates of the degree of proliferative activity have been derived. One is the mean daily increase of deoxypentosenucleic acid expressed as μg. P/g. wet wt. of nerve/day and the other is the mean daily increase of deoxypentosenucleic acid expressed as a percentage of the deoxypentosenucleic acid already present in the nerve. Both estimates show that, whereas the concentration of deoxypentosenucleic acid reached a maximum at 16 days, the peak in the rate of formation of deoxypentosenucleic acid was at 4 days. Fig. 3 also shows that the increase in the activity of the acid phosphomonoesterase (on a deoxypentosenucleic acid basis) is greatest at this same 4-day period, but that the increase in the activity of the β-glucuronidase, on a same basis, is still rising at 16 days.

It thus seems evident that in a degenerating nerve the increase in the β-glucuronidase activity per cell occurs later and persists longer than the cellular proliferation. In this respect the results are similar to those reported by Mills, Smith, Stary & Leslie (1950) and Mills (1951). These workers found that in the liver of the rat, regenerating after subtotal hepatectomy, the peak in the β-glucuronidase activity per unit deoxypentosenucleic acid occurred after the phase of rapid cellular proliferation.

The increase in the activity of β-glucuronidase in degenerating and regenerating nerves is also of interest in relation to the finding of Bernfeld & Fishman (1950) that electrophoretically pure β-glucuronidase from calf spleen was activated by nucleic acid. Deoxypentosenucleic acid was found to be more active than pentosenucleic acid and, subsequently, Bernfeld, Guarino & Fishman (1951) showed that thymidylic acid was active in this respect. As has been noted already, there is a great increase in the concentration of both types of nucleic acid in a degenerating nerve.

SUMMARY

1. The β-glucuronidase activity of the sciatic nerve of the cat increased greatly after either nerve section or nerve crush.

2. After nerve section the increase was statistically significant after 4 days (138%) and reached a maximum at 16 days (3400%). Thereafter it fell steadily.

3. After nerve crush the β-glucuronidase activity did not differ significantly from that observed after nerve section for the first 96 days. Even after 600 days the activity in the crushed nerves was significantly greater than that in the control nerves.

4. The β-glucuronidase activity per cell, calculated on the deoxypentosenucleic acid, increased to a maximum (1120%) at 16 days after nerve section while the peak in cellular proliferation was at 4 days.

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The Inhibition of Erythrocyte Cholinesterase by Tri-esters of Phosphoric Acid

2. DIETHYL *p*-NITROPHENYL THIONPHOSPHATE (E605) AND ANALOGUES

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In the first paper of this series (Aldridge & Davison, 1952) it was shown that inhibition of cholinesterase by a series of substituted diethyl phenyl phosphates increased as their stability to hydrolysis decreased. During this work it was found that many of the inhibitors contained a small amount of a more active inhibitor as an impurity and evidence was produced that this was tetraethyl pyrophosphate (TEPP).

Diggle & Gage (1951*a*) have recently shown that a chromatographically purified preparation of diethyl *p*-nitrophenyl thionphosphate (E605) has an extremely low inhibitory power against cholinesterase *in vitro*. In this laboratory this observation has been confirmed using the same sample (Aldridge & Barnes, 1952). However, many workers have stated that E605 is an active inhibitor of cholinesterase *in vitro* (Aldridge, 1950; DuBois, Doull, Salerno & Coon, 1949; Engbaek & Jensen, 1951; Grob, 1950; Hecht & Wirth, 1950; Metcalf & March, 1949; Sallé, 1950; Wirth, 1949). In a report of work carried out during the war years, Schrader (1951) has stated that E605 isomerizes upon distillation and devised a series of tests to determine the diethyl *p*-nitrophenyl thionphosphate content, its purity and freedom from *S*-ethyl isomer. In Table 1 are shown the formulae of the three possible isomers of E605. The whole question of the purity and *in vitro* activity of E605 against cholinesterase has been complicated by the fact that although pure E605 has a low inhibitory power *in vitro* it is still highly toxic to animals and is converted *in vivo* to a more active inhibitor of cholinesterase (Diggle & Gage, 1951*b*; Aldridge & Barnes, 1952). In this paper we have tried to determine the true *in vitro* activity of E605 and its isomers. This has entailed investigation into the purity of our specimens of inhibitors. The specimen of E605 previously used by one of us (Aldridge, 1950) has also been re-examined and it has been found that most of its inhibitory activity is due to diethyl *p*-nitrophenyl phosphate (E600).

A 'hydrolysis technique' which takes advantage of their different rates of hydrolysis in buffers (Aldridge & Davison, 1952) was developed for the