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Chemical Studies of Peripheral Nerve during Wallerian Degeneration

6. INCORPORATION OF RADIOACTIVE PHOSPHATE INTO PENTOSENUCLEIC ACID AND PHOSPHOLIPIN *IN VITRO*

BY W. L. MAGEE AND R. J. ROSSITER
Department of Biochemistry, University of Western Ontario, London, Canada

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In previous papers from this laboratory the changes that occur in the concentration of lipids (Johnson, McNabb & Rossiter, 1949; Burt, McNabb & Rossiter, 1950), nucleic acids (Logan, Mannell & Rossiter, 1952b), and certain enzymes (Hollinger, Rossiter & Upmalis, 1952; Hollinger & Rossiter, 1952) have been described for peripheral nerves degenerating after nerve section or regenerating after nerve crush. After nerve section there is a decrease in the concentration of the principal lipid components of the myelin sheath of the nerve fibre (myelin lipids) and a decrease in the concentration of the residue organic phosphorus (ROP), referred to as 'inositide P' by Logan, Mannell & Rossiter (1952a). There is also a smaller decrease in the concentration of 'phosphoprotein' phosphorus (PP) and an increase in the concentration of both deoxypentosenucleic acid (DNA) and pentosenucleic acid (PNA), associated with the cellular proliferation and the infiltration of macrophages that accompany the degeneration. If the nerve subsequently regenerates (after crush instead of section), the concentrations of myelin lipids, ROP and PP gradually return to normal and the concentrations of DNA and PNA decrease.

Since it has been shown that inorganic phosphate labelled with ^{32}P is incorporated into both the phospholipin (Fries, Schachner & Chaikoff, 1942; Schachner, Fries & Chaikoff, 1942; Strickland, 1954) and the PNA (DeLuca, Rossiter & Strickland, 1953; Findlay, Rossiter & Strickland, 1953) of brain slices respiring in a Krebs-Ringer bicarbonate medium containing glucose, it was decided to investigate the possible incorporation of ^{32}P into the lipids and PNA of normal, degenerating and regenerating peripheral nerve under similar conditions. In addition, observations were made on the incorporation of ^{32}P into the ROP and PP of normal, degenerating and regenerating nerve (see Strickland, 1952; Findlay, Strickland & Rossiter, 1954).

A preliminary report of some of these experiments has already appeared (Magee & Rossiter, 1954).

METHODS

The right sciatic nerve of a number of cats was either sectioned or crushed at the level of the greater trochanter of the femur. Details of the operations have been described previously (Johnson *et al.* 1949; Burt *et al.* 1950). No attempt was made to control the age, sex, or weight of the animals. After 8, 16, 32 or 96 days the animals were killed, and the segment of the nerve distal to the site of the operation 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 connective tissue, the epineurium was stripped off and the nerve was rapidly weighed. It was then cut into small lengths (about 1 mm.) and transferred to a Warburg flask containing Krebs-Ringer bicarbonate, glucose and [^{32}P]phosphate. The vessel was filled with a mixture of 95% O_2 and 5% CO_2 and incubated in a bath at 37·2° for 4 hr., with shaking to ensure proper oxygenation. Details of the incubation have been described by DeLuca *et al.* (1953).

After the incubation the nerve fragments were transferred to a mortar and the excess liquid was poured off. They were then frozen with liquid nitrogen and ground to a fine powder. This powder was added to the incubating fluid and mixed with 5 ml. cold 20% (w/v) trichloroacetic acid (TCA) to give a final concentration of 10% TCA. The tube and contents were kept at 0–4° by means of an ice bath. The acid-soluble P was removed as described by DeLuca *et al.* (1953). The residue was separated into lipid, PNA, ROP and PP and each fraction was purified as described by Findlay *et al.* (1954). The specific activity of the P in each purified fraction was then determined. The nucleic acids were extracted by the method of Hammarsten (1947), after which the PNA nucleotides were subjected to two magnesia treatments. For brain slices the mixed nucleotides so obtained have a specific activity within the range given by the individual nucleotides separated chromatographically (DeLuca *et al.* 1953). In experiments in which the nerves from a number of cats were pooled, the same was shown to be true for the nucleotides of the PNA of cat sciatic nerve.

All values were corrected for decay and calculated on the basis of a standard count of $8{\cdot}1 \times 10^5$ impulses/min./ml. incubating medium.

RESULTS

Normal nerves. Table 1 shows the specific activity of each of the four P-containing fractions of the normal sciatic nerve of the cat after incubation for 4 hr. in the medium containing glucose and [^{32}P]phosphate. In each experiment the right and the left sciatic nerve were incubated separately. The figures have not been subjected to any detailed statistical analysis, but it is evident that for each fraction the variation between animals is greater than the variation between the right nerve and the left nerve of the same animal. The table also shows that the specific activities of the P-containing fractions were in the order: PP > ROP > PNA > lipid P.

Table 2 shows the mean activity of each fraction expressed in terms of the number of impulses/min./100 mg. fresh nerve. The figures were calculated from the mean concentration of P in the fraction, as reported by Logan *et al.* (1952*b*), and the mean specific activity, as reported in Table 1. It can be seen that, although the specific activity of the PNA was considerably greater than that of the lipid P, the concentration was much less, so that per 100 mg. nerve, less ^{32}P was incorporated into the PNA than into the lipid. Also more ^{32}P was incorporated into the ROP, for this fraction had both a higher concentration and a higher specific activity than the PNA. Although the PP concentration was low, the specific activity of the fraction was very high, so that, in terms of fresh weight of nerve, the activity of the PP was of the same order as that given by the lipid P.

In Table 3 are reported the results of two experiments in which the specific activities were determined either before or after the incubation period. The 4 hr. values are for a nerve incubated in the usual way. The 0 hr. values are for the sciatic nerve from the opposite side of the body. This nerve was prepared as usual and placed in the vessel containing the medium and the [^{32}P]phosphate. The vessel was shaken to ensure thorough mixing and the nerve fragments were then removed and frozen. The proteins were precipitated with TCA in the presence of the [^{32}P]phosphate, as was customary for the in-

Table 1. *Specific activity of the phosphorus compounds of the sciatic nerve of the cat*

Nerve fragments incubated at 37·2° in Krebs–Ringer bicarbonate containing [^{32}P]phosphate. Glucose, 0·011 M. Incubation time, 4 hr. Counts/min./µg. P.

Expt. no.	Lipid P Right nerve	Lipid P Left nerve	PNA Right nerve	PNA Left nerve	ROP Right nerve	ROP Left nerve	PP Right nerve	PP Left nerve
1	6·1	6·9	63	72	104	146	1070	1270
2	10·8	10·8	90	114	204	241	1030	1200
3	6·3	5·8	89	92	119	80	900	810
4	10·8	9·8	116	94	217	160	1600	1440
5	8·4	8·6	91	76	166	166	1890	1930
6	7·5	8·5	—	—	125	136	1470	1860
7	8·2	8·9	125	150	179	247	1230	1350
Mean	8·4		98		164		1360	

Table 2. *Activity of the phosphorus compounds of sciatic nerve of the cat*

	Concn. (µg. P/100 mg. wet tissue ±S.E.M. From data of Logan *et al.* 1952*b*)	Mean specific activity (counts/min./µg. P. From Table 1)	Activity (counts/min./100 mg. wet tissue)
Lipid P	310±5·6	8·4	2600
PNA	3·9±0·1	98	380
ROP	9·0±0·4	164	1500
PP	1·8±0·1	1360	2400

cubated nerves. The table shows that only small amounts of the ^{32}P were incorporated into the 0 hr. nerve. These findings indicate that the purification procedure for each of the fractions was satisfactory. They also suggest that the incorporation of ^{32}P into each of the fractions is a metabolic phenomenon.

Strickland (1954) and Findlay *et al.* (1953) reported similar results for the lipid P and PNA of brain slices. These workers also showed that the incorporation of ^{32}P was abolished by anaerobic conditions, by the addition of metabolic inhibitors, or by the addition of 2:4-dinitrophenol in concentrations that do not decrease the oxygen uptake. It thus seems likely that the incorporation of ^{32}P into each of the fractions is dependent upon the existence of oxidative phosphorylation processes within the nerve.

Sectioned nerves. Nerves degenerating for different periods of time after nerve section were incubated for 4 hr. in the medium containing glucose and [^{32}P]phosphate. Table 4 shows the change in the mean specific activity of each fraction. The specific activities of the degenerating nerves are expressed as percentages of the specific activities of the control nerves from the same animals.

After nerve section the specific activity of the lipid P increased with each successive period of degeneration, to reach a value greater than 12 times that of the control nerve by the end of 96 days. In contrast, the specific activity of the PNA decreased during the early stages of the degeneration, falling to 60 % of that of the control nerves by the end of 16 days. At the 32- and 96-day periods the specific activity of the PNA of the sectioned nerves did not differ significantly from that of the controls.

The specific activity of the ROP fraction, like that of the PNA, was less than normal at 8 and 16 days after the operation. The specific activity of this fraction then commenced to increase. By 32 days it did not differ significantly from that of the control nerves, and by 96 days it was significantly greater. The only time interval at which the specific activity of the PP was greater than that of the controls was at 96 days.

Table 3. *Specific activity of the phosphorus compounds of the sciatic nerve of the cat before and after incubation*

Nerve fragments incubated at 37·2° in Krebs–Ringer bicarbonate containing [^{32}P]phosphate. Glucose, 0·011 M. Counts/min./μg. P.

	Expt. 1		Expt. 2	
	0 hr.	4 hr.	0 hr.	4 hr.
Lipid P	0·5	8·4	0·4	7·7
PNA	2	113	2	148
ROP	3	178	5	203
PP	70	1130	57	1170

Table 4. *Effect of nerve section or nerve crush on the specific activity of the phosphorus compounds of the sciatic nerve of the cat*

Incubation conditions as in Table 1. Results are expressed as (specific activity degenerating nerve ÷ specific activity control nerve) × 100. No. of animal is stated in parentheses under each result. *P* gives the significance of the change reported.

Time after section (days)	Lipid P Mean	S.E.M.	*P*	PNA Mean	S.E.M.	*P*	ROP Mean	S.E.M.	*P*	PP Mean	S.E.M.	*P*
A. Nerve section												
8	132	± 15 (4)	<0·1	80	± 8 (4)	<0·05	54	± 5 (4)	<0·01	88	± 8 (4)	>0·3
16	226	± 24 (10)	<0·01	61	± 4 (6)	<0·01	55	± 8 (10)	<0·02	95	± 9 (9)	>0·4
32	678	± 106 (3)	<0·05	100	± 18 (3)	>0·9	104	± 40 (3)	>0·8	108	± 42 (3)	>0·7
96	1290	± 64 (7)	<0·001	121	± 20 (6)	>0·6	173	± 18 (7)	<0·01	156	± 22 (7)	<0·05
B. Nerve crush												
Time after crush (days)												
32	548	± 73 (4)	<0·02	104	± 15 (4)	>0·9	130	± 52 (3)	>0·7	84	± 28 (3)	>0·4
96	540	± 56 (6)	<0·001	112	± 18 (6)	>0·3	209	± 22 (6)	<0·05	165	± 23 (6)	>0·1

Table 5. *Effect of nerve section or nerve crush on the activity of the phosphorus compounds of the sciatic nerve of the cat*

	Lipid P			PNA		
Time after section (days)	Mean concn. (mg. P/100 g. wet tissue ±S.E.M.)	Mean specific activity (counts/min./μg. P)	Activity (counts/min./100 mg. wet tissue)	Mean concn. (mg. P/100 g. wet tissue ±S.E.M.)	Mean specific activity (counts/min./μg. P)	Activity (counts/min./100 mg. wet tissue)
			A. Nerve section			
0	310±5·6	8·4	2600	3·9±0·1	98	380
8	293±22	11·1	3250	14·8±0·7	79	1170
16	156±20	19·0	3000	18·5±0·8	60	1110
32	79±10	52	4100	20·8±2·5	98	2000
96	22±2	108	2400	9·8±0·8	119	1170
Time after crush (days)			*B.* Nerve crush			
32	78±12	46	3600	17·0±2·4	103	1750
96	110±9	45	4950	9·0±1·2	110	990

The effect of nerve section on the concentration of the four fractions under study was reported by Logan *et al.* (1952*b*). Table 5 shows the mean concentrations and the mean specific activities of the lipid P and PNA at each of the time intervals after nerve section. From these figures has been calculated the activity of each of the fractions in terms of the number of impulses/min./100 mg. wet nerve. The activity of the lipid P/100 mg. nerve increased with each successive time period after nerve section up to 32 days, and then decreased by 96 days. For the PNA, the activity/100 mg. nerve was greater than that of the control nerves at all time intervals after section. The activity/100 mg. reached a maximum at 32 days, at which time the concentration of PNA was at its greatest and the specific activity had returned to normal.

Crushed nerves. Table 4 also shows the results for nerves regenerating 32 or 96 days after nerve crush. At 32 days after the operation the specific activity of no fraction differed significantly from that of the corresponding fraction 32 days after nerve section. The specific activity of the lipid P was significantly greater than that of the lipid P of the control nerves, but the specific activity of each of the other fractions did not differ significantly from the values for the intact controls.

At 96 days the specific activity of the lipid P of the crushed nerves, although still much greater than that of the controls, was significantly less than that of the sectioned nerves. Table 5 shows that at this time interval considerable re-myelination had taken place, as demonstrated by the increase in the concentration of lipid P. At 32 days after nerve crush the figures for the activity of the lipid P/100 mg. nerve were not significantly different from those reported for nerve section, but by 96 days, the activity of the regenerating nerves had become more than twice that of the degenerating sectioned nerves.

The changes observed after nerve crush in both the specific activities and the activities/100 mg. nerve for the PNA, ROP and PP fractions did not differ significantly from the corresponding values reported after nerve section (Tables 4 and 5).

By 96 days after the operation both the concentrations and the specific activities of the lipid P and PNA were returning towards the values given by the intact nerves (Table 5). By this time axons from the undamaged portion of the nerve proximal to the site of injury had grown down into the peripheral portion, and re-myelination was proceeding. It is of interest to note that the activity of the lipid P/100 mg. nerve was higher 96 days after nerve crush than at any other period studied.

Are the results caused by a barrier limiting the inward diffusion of inorganic ^{32}P *into the nerve?*

Feng & Gerard (1930) found that for the sciatic nerve of the frog the connective tissue sheath surrounding the nerve may act as a diffusion barrier for certain inorganic ions. The experiments of Mullins & Grenell (1952) with frog nerve and Causey & Palmer (1953) with rabbit nerve demonstrated that the epineural sheath functions as a diffusion barrier for inorganic ^{32}P. In our experiments the epineurium and surrounding connective tissue were stripped off the nerve and the remainder of the nerve was then cut into small pieces approximately 1 mm. in length. Before the nerve was cut up in this way it would correspond to the stripped nerve of Causey & Palmer (1953).

From Table 5 it can be seen that in the degenerating nerves the specific activity of the lipid P increased, and the concentration of the lipid P decreased, with each successive time period after nerve section. Table 5 also shows that during the early stages of the degeneration the specific activity of the PNA fell and the concentration of PNA

increased. These observations raised the possibility that during the incubation period some diffusion barrier within the nerve might limit the inward diffusion of inorganic ^{32}P to a constant amount and that only this amount might be available for incorporation into the P-containing fractions. Other conditions remaining the same, the specific activity of each fraction would then be determined by the P concentration of that fraction, a reduction in the concentration of P of the fraction causing an increase in the specific activity, and vice versa.

An examination of the figures presented in Table 5 shows that the incorporation of ^{32}P into either the lipid P or the PNA is not the same for each time interval after nerve section, even on a nerve-weight basis. Also, the relative changes in the activities for PNA are quite different from those for lipid P. These observations would be difficult to explain solely in terms of the restricted penetration of ^{32}P. In addition, there are other observations inconsistent with such an explanation, i.e. the finding that the peak in the concentration of PNA was at 32 days, whereas the minimum in the specific activity was at 16 days, and the finding that both the concentration (Logan *et al.* 1952*b*) and the specific activity of the ROP fell during the early stages of the degeneration.

Despite the fact that precautions were taken to remove any diffusion barrier, by the stripping of the epineurium and the cutting of the nerve into short lengths, it was decided, as a further check, to investigate the incorporation of ^{32}P into finely divided homogenate preparations. Since cat sciatic nerve could not be homogenized satisfactorily, the sciatic nerve of the rat was used for these experiments. The specific activities were studied in a suitably 'reinforced' system, similar to that used by Strickland, McMurray & Rossiter (to be published) for rat brain.

The sciatic nerve of each of two rats was sectioned on one side. After 20 days both intact and degenerating nerves were removed and homogenized in a homogenizer of the Potter–Elvehjem type. The pooled normal and pooled degenerating nerves were incubated in a medium containing (in final concentrations) the following: glucose, 0·028 M; hexose diphosphate (K salt), 0·005 M; adenosine triphosphate (K salt), 0·007 M; coenzyme I, 0·001 M; cytochrome *c*, 0·00006 M; potassium fumarate, 0·0016 M; nicotinamide, 0·04 M; $MgCl_2$, 0·008 M; glycylglycine buffer (pH 7·4) 0·083 M; phosphate buffer (pH 7·4), 0·01 M. Sufficient [^{32}P]phosphate, made up in 0·1 ml. neutralized buffer, was added to make the specific activity of the inorganic P of the incubating medium the same as that in the cat-nerve experiments. The vessels were filled with oxygen and the homogenates were incubated, with shaking, for 1 hr. The specific activities of the lipid P and PNA were then determined as described previously.

Table 6. *Specific activity of the phosphorus compounds in a homogenate of the sciatic nerve of the rat*

Homogenate incubated as described in text. Counts/min./μg. P.

	Expt. 1		Expt. 2	
	Control	20 days after section	Control	20 days after section
Lipid P	0·7	3·3	0·5	2·1
PNA	4·9	1·7	—	—

Table 6 shows that with such a system the specific activity of the lipid P was much greater 20 days after nerve section, whereas the specific activity of the PNA was less. These results, which are similar to those reported in Table 4 for fragments of cat nerve, provide additional evidence that the changes in specific activity are metabolic, and that they are not due to a restricted penetration of the inorganic ^{32}P into the nerve.

DISCUSSION

The finding that ^{32}P was incorporated into the lipid P of cat sciatic nerve respiring in a glucose-containing medium *in vitro* agrees with the previous report of Fries *et al.* (1942) for dog nerve. The demonstration of such an incorporation suggests that the phospholipins of the myelin sheath may be formed *in situ* and neither taken up from the blood stream as such, nor formed in the body of the nerve cell and passed down to the myelin sheath of the nerve fibre.

The experiments also indicate that ^{32}P is incorporated into the lipid P, PNA, ROP and PP of peripheral nerve. They are thus complementary to the previous findings that ^{32}P is incorporated into the corresponding fractions of cat brain slices (DeLuca *et al.* 1953; Strickland, 1954; Findlay *et al.* 1954). It is of interest to note that with cat sciatic nerve the figures are of the same order as those found in previous experiments with cat brain slices for lipid P, ROP and PP, but that for PNA the sciatic nerve figures are considerably greater (Findlay *et al.* 1954).

Lipid phosphorus. The increase in the specific activity of the lipid P in the degenerating nerves is in accord with the findings of Bodian & Dziewiatkowski (1950) and Samuels, Boyarsky, Gerard, Libet & Brust (1951). These workers administered [^{32}P]phosphate to monkeys and guinea pigs and reported a greater *in vivo* incorporation of the ^{32}P into the lipid P of the sectioned nerves than into the lipid P of intact nerves. Although such experiments *in vivo* might be considered more physiological, their interpretation is complicated by the possibility that the ^{32}P incorporation into the degenerating nerve might be affected by changes in the blood flow and by changes in the permeability of the blood vessels supplying the nerve. The blood flow could be

modified by changes in both the number and in the degree of dilation of these vessels. In spite of these complicating factors, there was an increase in the specific activity of lipid P during Wallerian degeneration both *in vivo* and *in vitro*.

An interpretation of the results in terms of existing knowledge of the chemistry of Wallerian degeneration is difficult. At the time of myelin destruction, when the concentration of lipid P is decreasing, more ^{32}P is incorporated into the lipid during the *in vitro* incubation. Several explanations are possible:

(i) As Wallerian degeneration proceeds, the myelin sheath breaks up physically, enabling the phospholipins of the myelin sheath to come into closer proximity to the inorganic ^{32}P, or some labelled P-containing precursor. The finding that there was an increase in the specific activity of the lipid P of an homogenate of rat nerve is evidence against this explanation.

(ii) The enzymes that catalyse the exchange between inorganic ^{32}P and the P of the phospholipin are present in Schwann cells and macrophages. These cells become more numerous and more active as the nerve degenerates, and so the concentration of enzymes in the *in vitro* system increases, reaching a maximum in 32 days. At 96 days after nerve section, when the specific activity of the lipid P is still very high, the cellularity of the nerve has greatly decreased (Logan *et al.* 1952*b*), but the cells are still metabolically active, as is evidenced by the high specific activity of the PNA.

(iii) The phospholipin of the myelin sheath is metabolically inert in so far as the incorporation of ^{32}P is concerned, and the metabolically active lipids are in cells such as the Schwann cells and macrophages. These cells increase in number during the course of the degeneration and it may be non-myelin lipid in these cells, rather than the rapidly disappearing myelin lipid, that takes up the ^{32}P. It is possible that the ^{32}P is incorporated into the individual phospholipins at different rates. The recent reports of Dawson (1953) and Streicher (1953) on the specific activities of the individual phospholipins of brain homogenates are particularly important in this regard.

Protein-bound phosphorus. For technical reasons our PNA, ROP and PP fractions cannot be compared with the fractions studied by Bodian & Dziewiatkowski (1950) and Samuels *et al.* (1951) in their experiments *in vivo*. The 'residual P' of Bodian & Dziewiatkowski (1950) included PNA, DNA, PP and our ROP, and the 'nucleic acid' of Samuels *et al.* (1951) included PNA, DNA and a considerable portion of our ROP. Since each of these components has a greatly different specific activity, and since the components vary greatly in their relative concentrations during Wallerian degeneration (Logan *et al.* 1952*b*), the specific activity of these fractions would not be expected to bear any simple relation to the specific activity of the PNA, as reported here.

The specific activity of the PNA was significantly decreased in the degenerating nerve at both 8 and 16 days after nerve section (Table 4). However, at all time intervals after section the activity/100 mg. nerve was greater for the PNA (Table 5). During the degeneration there was an increase in the cellularity of the nerve, as judged by the concentration of PNA.

The ROP fraction is of some interest. The specific activity of this fraction was less than that of the control nerves at both 8 and 16 days after nerve section (Table 4). Logan *et al.* (1952*a*, *b*) have suggested that the phosphorus of this fraction may be derived, in a large measure, from the phosphorus of the inositol-containing lipid-protein complex described by Folch & LeBaron (1951), and thought by them to be part of the classical neurokeratin of Kühne & Chittenden (1889). That the concentration of this fraction decreases during Wallerian degeneration is evidence in favour of this view. However, Middleton (to be published) has shown that this fraction, like the concomitant P of liver tissue described by Davidson & Smellie (1952), is comprised of a number of highly active P-containing compounds.

Little is known of the origin of the PP. The specific activity of the PP fraction is very high (Table 1), but the concentration is quite low. By 96 days after nerve section the specific activity of this fraction was greater than that of the controls.

Regenerating nerves. At 32 days neither the specific activity nor the concentration of any of the P-containing fractions of nerves regenerating after nerve crush differed significantly from the corresponding figures for nerves degenerating after section. The only difference observed in the regenerating nerves was for the lipid P at 96 days after the operation. At this time there was a significant increase in the concentration of lipid P and a decrease in its specific activity. However, the activity of the lipid P/100 mg. nerve was significantly increased. This increase is probably related to the production of new myelin that is occurring at this time.

SUMMARY

1. Inorganic phosphate labelled with ^{32}P was found to be incorporated into the lipid P, pentose-nucleic acid (PNA), residue organic P (ROP) and 'phosphoprotein' (PP) of the sciatic nerve of the cat respiring in a Krebs–Ringer bicarbonate medium containing glucose and [^{32}P]phosphate.

2. The specific activities of the fractions were in the order: PP > ROP > PNA > lipid P.

3. In nerves degenerating after section and incubated under the same conditions, there was a large increase in the specific activity of the lipid P. During the early stages of the degeneration (8 and 16 days after nerve section) there was a decrease in the specific activity of the PNA and ROP. At 8, 16 and 32 days after nerve section the activity of the lipid P and PNA/100 mg. nerve was greater than the corresponding figure for control nerves.

4. In regenerating nerves 32 days after nerve crush, the specific activity of each of the four P-containing fractions did not differ significantly from that of the corresponding fraction 32 days after nerve section. By 96 days after the operation the specific activity of the lipid P was significantly less than that of the lipid P of nerves at the same time interval after section. The activity/100 mg. nerve was considerably greater.

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Actinomycete Metabolism: Origin of the Guanidine Groups in Streptomycin

BY G. D. HUNTER,* MARY HERBERT AND D. J. D. HOCKENHULL
Glaxo Laboratories, Ltd., Sefton Park, Stoke Poges, Bucks

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Despite the great interest that streptomycin has evoked in recent years, very little is known about the biochemical pathways leading to its production by *Streptomyces griseus*. Karow, Peck, Rosenblum & Woodbury (1952) have demonstrated the incorporation of the labelled carbon of [^{14}C]glucose into streptomycin. They did not, however, submit their product to chemical degradation, so that the distribution of the radioactivity between the various parts of the molecule is unknown. Nor is it clear from their results whether glucose functions as a direct precursor of the trisaccharide molecule or is first degraded into smaller molecular fragments.

Our first points of attack have been the guanidine side chains of the streptomycin molecule and the results set out here show that the carbon atoms concerned are derived largely, if not entirely, from carbon dioxide. The mode of biosynthesis of the guanidine groups as a whole does not present as clear a picture, but there is an indication that arginine takes part in the process.

EXPERIMENTAL

Fermentation. The organism, *Streptomyces griseus, albus* mutant (Dulaney, Z38) is described in the patent covering its production (Dulaney, 1951). The development of a vegetative inoculum in a special meat medium and its use to inoculate a soybean medium are also described in example 1

* Present address: The Chester Beatty Research Institute, Pollard's Wood, Chalfont St Giles, Bucks.