THE SPREAD OF A NEUROTROPIC STRAIN OF HERPES VIRUS IN THE CEREBROSPINAL AXIS OF RABBITS

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THE question whether a neurotropic strain of herpes virus spreads within the nervous system along axis cylinders or within tissue fluid spaces was first raised almost simultaneously by Marinesco and Draganesco (1923) and by Goodpasture and Teague (1923–24). The former regarded the intercellular spaces as the more probable pathway for the centripetal movement of the virus, whereas the latter believed that it gained early access to the peripheral nerve fibres and was conducted within the confines of the axons to their motor or sensory nuclei in the central nervous system. Recently Field (1952) has reconsidered the problem by again employing histological methods to identify the sites of inflammation in the trigeminal nerve trunk and the brain stem resulting from the inoculation of the virus into the cornea and masseter muscle of rabbits. From his findings, he has gained the impression that they tend to support the hypothesis of " lymphatic space progression rather than that of axonal transmission."

Few attempts have yet been made to identify sites of experimentally induced inflammation in the central nervous system by making use of the local escape of some recognisably marked colloid in spite of the fact that the normally low permeability of its capillary walls to large molecules renders this organ particularly well suited to the employment of such techniques. In their study on the invasion of the brain stem by herpes virus inoculated into the territory of the fifth cranial nerve, McClellan and Goodpasture (1923–24) injected trypan blue into the circulating blood and observed its extravasation at the site of inflammation. Later, Faber (1936–37) used the same procedure for identifying the sites of pre-paralytic lesions in monkeys in which poliomyelitis had been induced by intranasal infection. Although vital dye techniques display the foci of inflammation conspicuously to the naked eye, any attempt to apply them quantitatively, such as that devised by Sachs and Lummis (1955), has the serious disadvantage that it necessarily entails the destruction of the specimen and thus precludes any subsequent comparative histological examination of the tissues.

The introduction of radioactive isotopes for the labelling of proteins has not only extended the applicability of this method of detecting sites of injury through the focal accumulation of a marked colloid, but it has placed it on a better quantitative basis than any possible with vital dyes. The present paper describes the use of radio-iodinated homologous serum proteins for identifying the location and assessing the severity of the inflammatory reactions excited in the cerebrospinal axis of rabbits by the injection of a neurotropic strain of herpes virus into three widely spaced peripheral spinal nerve trunks. Its conclusions may be anticipated

by stating that the segmental distribution of the subsequent inflammatory reactions in the spinal cord appear to be more compatible with a dispersal of the virus from its point of entry into the neuraxis by way of its tissue fluid spaces rather than along its axis cylinders.

METHODS

Animals

All observations were made on Copenhagen White rabbits weighing 1·5–2·5 kg.

Virus

The Brussels strain of neurotropic herpes virus used was kindly given by Dr. Pierre Lépine. It was maintained by passage in the central nervous system of rabbits, in some by intracerebral inoculation and in others by direct inoculation into the lumbar spinal cord. These latter preparations were the more potent. The infected nervous tissue from the moribund animals was triturated with four times its volume of ice-cold sterile broth and preserved in the freeze-dried state in small ampoules in volumes of 0·1 ml. Re-suspension was effected by the addition of an equal volume of sterile water. When injected intracerebrally, these preparations killed rabbits in 3–6 days.

Operative procedures

Before operation, the rabbits were anaesthetised with " veterinary Nembutal " (0·4 ml./kg.) given intravenously and followed by open ether. The sciatic, median or first lumbar spinal nerves were exposed with aseptic precautions, and the virus suspension was injected directly into the selected nerve trunk with the aid of a micrometer-controlled 0·5 ml. tuberculin syringe. The volumes injected were about 40, 30 and 20 μl. respectively. The wounds were closed with silk sutures and covered with a protective layer of collodion.

Post-operative clinical observations

Each animal was examined at least once daily for evidence of pyrexia, paralysis and changes in knee and " splay " reflexes (see Gutmann, 1942).

Preparation of radioactive protein

Rabbit plasma proteins labelled with ^{131}I were prepared by the method described by Veall, Pearson and Hanley (1955). The protein solution was injected under light Nembutal anaesthesia into the marginal ear vein 20 hr. before the animal was killed. The dosage usually employed was 400 μc./kg. in a volume of 1–3 ml./kg. About 40 per cent of this labelled protein remained in the circulating blood at the end of 20 hr.

Preparation of specimens from the central nervous system

Immediately before death a sample of blood was taken from the marginal ear vein for the subsequent determination of the terminal plasma radioactivity. The rabbits were killed with Nembutal and at once eviscerated. The brain and spinal cord were then exposed *in situ*, and the carcass immersed for 48 hr. in 10 per cent formol-saline. After fixation, the entire central nervous system was removed *en bloc*, care being taken to trim away all extramedullary nervous tissue. The spinal cord between the levels of the lower border of the cerebellum and the third sacral nerve roots was divided into 17 portions of equal length. In most rabbits this mode of division gave portions of cord 2 cm. in length and weighing between 150 and 400 mg. The relationship of these 17 portions to the anatomical segments of the spinal cord was as follows :

Portion number.	Segment.	Portion number.	Segment.	Portion number.	Segment.
1	C 1 and 2	7	T 7 and 8	13	L 3
2	C 3 and 4	8	T 9 and 10	14	L 4 and 5
3	C 5 and 6	9	T 11	15	L 6
4	C 7 and 8 ; T 1	10	T 12	16	L 7 ; S 1
5	T 2, 3 and 4	11	L 1	17	S 2 and 3
6	T 5 and 6	12	L 2		

The small terminal portion of the spinal cord (No. 17), the conus medullaris, yielded radioactivity values both higher and more variable than those of the remaining sixteen—a difference attributable to the proportionately larger contribution of irremovable nerve rootlets at this level. For this reason, this portion was discarded.

After the separation of the cerebral hemisphere and cerebellum, the rest of the brain was divided into four roughly equal portions : the remainder of the medulla oblongata (M), the pons (P) and the regions of the corpora quadrigemina (Q) and the basal ganglia (B).

Each specimen, after light blotting with filter paper, was weighed promptly with particular care to avoid desiccation and placed immediately in a test tube (80 × 12 mm.) containing 4 ml. of formol-saline.

Estimation of radioactivity

Measurements of gamma-ray activity were made with an apparatus of the type described by Veall and Baptista (1954), modified for use with a smaller specimen by reducing the number of G4Pb tubes to four and mounting them symmetrically as closely as possible to the centrally placed sample tube. This apparatus had a sensitivity for ^{131}I of 7500 c.p.m./ μc., and a background of 20 c.p.m.

Each specimen was counted for two periods of 5 min. each, and an average value obtained. From this and from the weight, the corrected c.p.m./g. was calculated. In order to achieve comparability between the various animals used, this latter figure was in each case related to the corresponding terminal level of plasma radioactivity. Thus, by dividing the c.p.m./g. for each specimen by the plasma c.p.m./100 μl. obtained for the respective animal, an index, the " plasma equivalent," was derived. In this way, allowance was made for the individual differences in the plasma radioactivity of the various rabbits.

RESULTS

Control Animals

The levels of radioactivity developing in the various designated portions of the central nervous system as a result of the intravenous injection of the labelled plasma proteins were determined for four rabbits. From Fig. 1, it can be seen

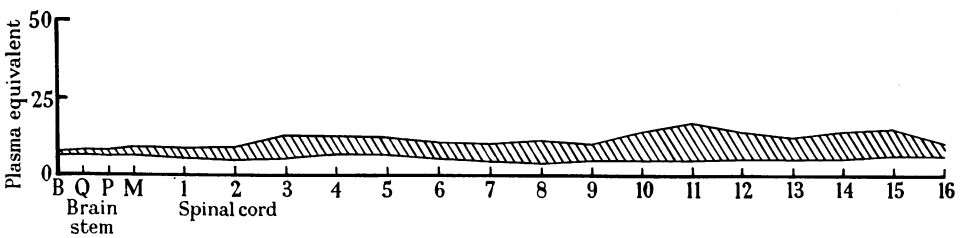

FIG. 1.—Limits of radioactivity (expressed as plasma equivalents) of consecutive portions of the brain stem (B., Q., P. and M.) and spinal cord (No. 1 to 16) of four control rabbits which had been injected intravenously 20 hr. before death with ^{131}I-labelled homologous plasma proteins.

that there was little variation in the plasma equivalent amongst the twenty consecutive portions into which the neuraxis from the basal ganglia to the lumbar enlargement had been divided, and that their radioactivity could be accounted for by a contained amount of plasma equivalent to about 1 per cent of their volume. Since the capillaries of the central nervous system are normally relatively impermeable to colloids, it seems likely that the greater part of these labelled proteins was present in the small blood vessels whose contents were less likely than those of

the larger and more superficial ones to be drained during the process of preparing the specimens.

Infected Animals

Clinical course

Infected rabbits usually developed pyrexia of from 104·5–106·5° F. (rectal temperature) on the 5th or 6th day after an intraneural inoculation. Nervous disturbances appeared 1–3 days later. After an inoculation into the median or sciatic nerves, paresis of the injected limb was invariably the first sign of involvement of the spinal cord. Soon, the opposite limb was also affected, and the full syndrome of fore- or hind-quarters paralysis quickly followed. After an injection into the first lumbar spinal nerve, paresis of the limbs appeared late and always initially in the hind limbs; sagging of the belly wall from weakness of the abdominal muscles was sometimes observed. The " splay reflex " described by Gutmann (1942) was usually lost after either a sciatic or first lumbar spinal nerve inoculation, but the knee jerks were consistently abolished only after the injection of the former. Moribund animals were usually markedly hypothermic and showed severe generalised muscular weakness. None of the rabbits injected intraneurally presented the typical syndrome of herpes encephalitis (fits, grinding of teeth and profuse salivation).

The animals were generally killed one, sometimes two or three, days after the onset of definite nervous disturbances: thus the labelled protein was usually given on the day on which unequivocal signs of injury to the spinal cord had become apparent.

Gross appearances

The spinal cords from infected rabbits usually showed superficial haemorrhages distributed in a characteristic manner. The largest were always on the dorsal aspect at the spinal level and on the same side as the incoming sensory roots corresponding to the nerve trunk injected. There were often further smaller superficial haemorrhages in the adjacent portions both above and below that primarily affected; these were sometimes on the opposite dorsal aspect of the spinal cord, particularly those remote from the level of main injury. Haemorrhages were rarely present on the ventral aspect of the cord.

A patchy meningeal exudation, unrelated to the sites of the main haemorrhagic lesions, was sometimes found, particularly in the sacral region. The more severely affected portions were often swollen and friable.

Segmental distribution of radioactivity

The radioactivity determinations of the affected portions of the spinal cord were substantially and consistently raised: at their maximum, the plasma equivalents were some 10–12 times higher than those found for the corresponding portions in the control animals. Moreover, the highest values in each animal were found in the portions segmentally related to the nerve trunk injected. Although the plasma equivalents in the most severely affected portions reached maximal values closely similar for all three sites of intraneural injections used, the curves for the distribution of the radioactivity along the cerebrospinal axis showed characteristics distinctive for each. The individual records for the four rabbits in

each of the three groups injected by the median, first lumbar spinal and sciatic nerves are set out in Fig. 2, 3 and 4 respectively. The mean values for each of the three groups are shown in Fig. 5.

Median nerve.—The central connexions of the brachial plexus lie in portions 3 and 4 of the spinal cord. Of the rabbits injected through the median nerve, the maximal radioactivity was found in portion 3 in two rabbits and in portion 4 in the other two. The mean values for all four rabbits, shown in Fig. 5, indicate that these portions are almost equally affected. From these maxima, the radio-

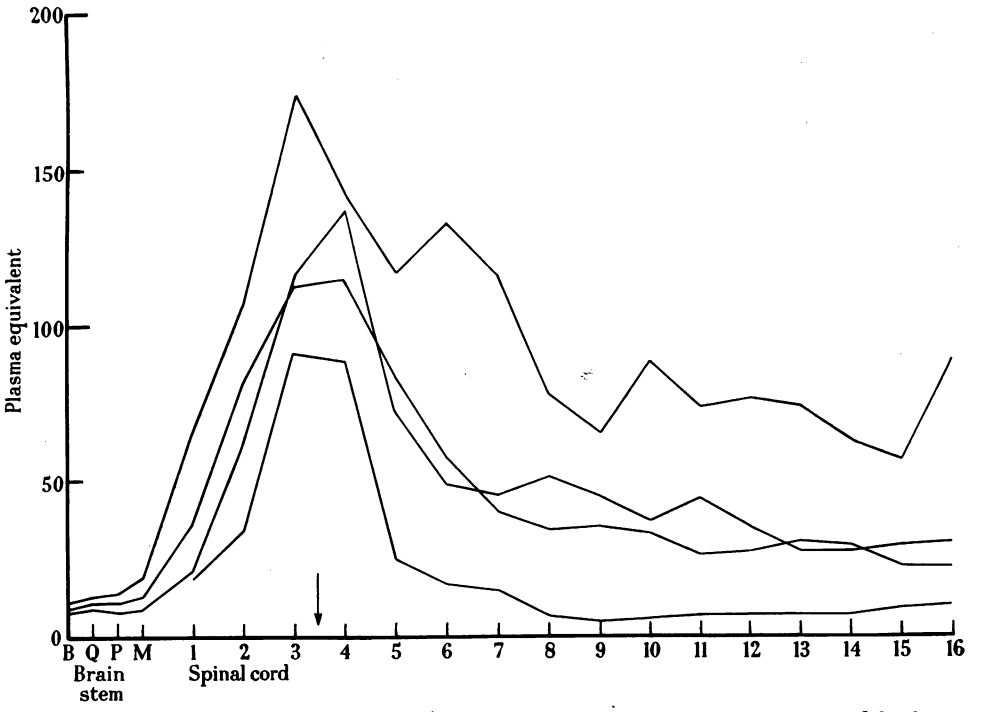

FIG. 2.—Radioactivity of the cerebrospinal axis of four rabbits which had been injected firstly with herpes virus into the median nerve and secondly with ^{131}I-labelled proteins intravenously. The arrow marks the spinal level of the nerve inoculated.

activity fell sharply in the cephalad direction, reaching values at the junction of the spinal cord and medulla oblongata that were little larger than those observed in the control rabbits, while in the caudal direction, significantly raised values were found far down in the thoracic and even in the lumbar segments. It would seem therefore that the agent which provokes the inflammation spreads more easily downwards than upwards in this region of the spinal cord. This difference might be associated with the relative fixity of the cord to surrounding structures near the base of the skull and its greater mobility within the vertebral canal in the thoracic and lumbar regions.

First lumbar spinal nerve.—This nerve arises from a segment included in portion 11, and from the curves depicting radioactivity given in Fig. 3, it can be seen that in all four animals in this group the peak lay either in this portion or

in those immediately above or below it. From this maximum, as can be seen from the curve for mean values given in Fig. 5, the fall is almost symmetrical, the abnormally elevated radioactivity extending downwards into the sacral region and upwards into the cervical region as far as the medulla. In this mobile region of the spinal cord, the agent responsible for the myelitis appears to be carried with equal facility in either direction.

Sciatic nerve.—This nerve trunk is composed of elements derived mainly from the 7th lumbar and 1st sacral segments, both of which lie in portion 16 of the spinal

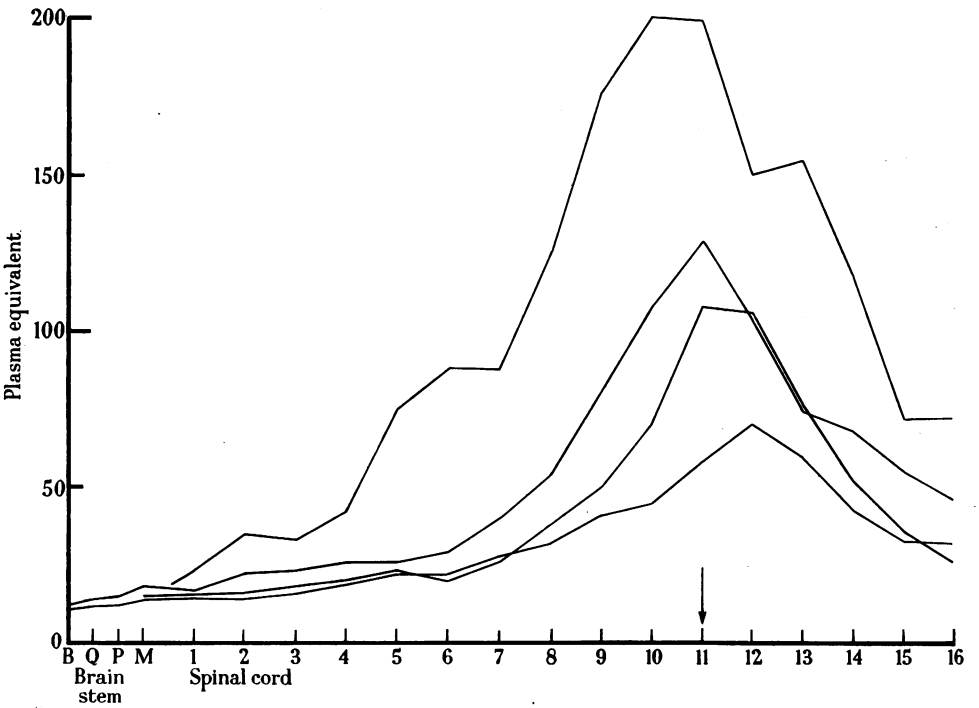

FIG. 3.—Radioactivity of the cerebrospinal axis of four rabbits which had been injected firstly with herpes virus into the first lumbar spinal nerve and secondly with ^{131}I-labelled proteins intravenously. The arrow marks the spinal level of the nerve inoculated.

cord. As can be seen from Fig. 4, the highest radioactivity was usually in this portion, although in one rabbit the maximum occurred two portions higher. It is evident from the mean curve in Fig. 5, that the radioactivity declined in a roughly logarithmic manner in a cephalad direction to reach values in the lower part of the thoracic spinal cord little above those found in the corresponding portions of the control animals.

Histological appearances

In order to compare alterations in radioactivity at various levels with microscopical evidence of structural injury, histological sections were prepared from each portion of the infected spinal cords. In brief it may be stated that the changes

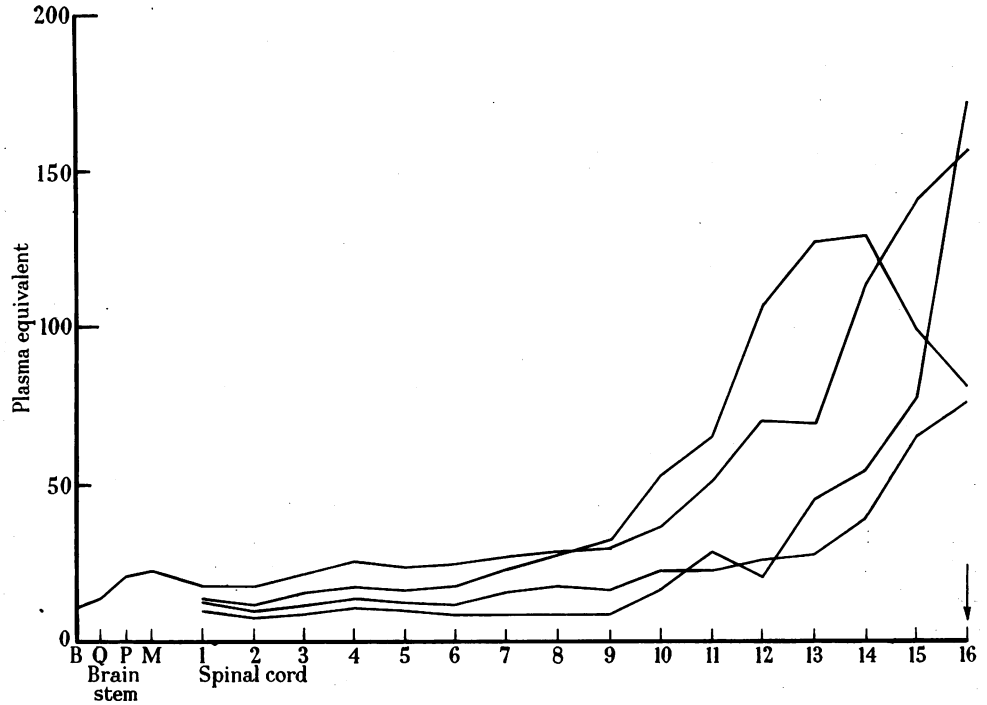

FIG. 4.—Radioactivity of the cerebrospinal axis of four rabbits which had been injected firstly with herpes virus into the sciatic nerve and secondly with ^{131}I-labelled proteins intravenously. The arrow marks the spinal level of the nerve inoculated.

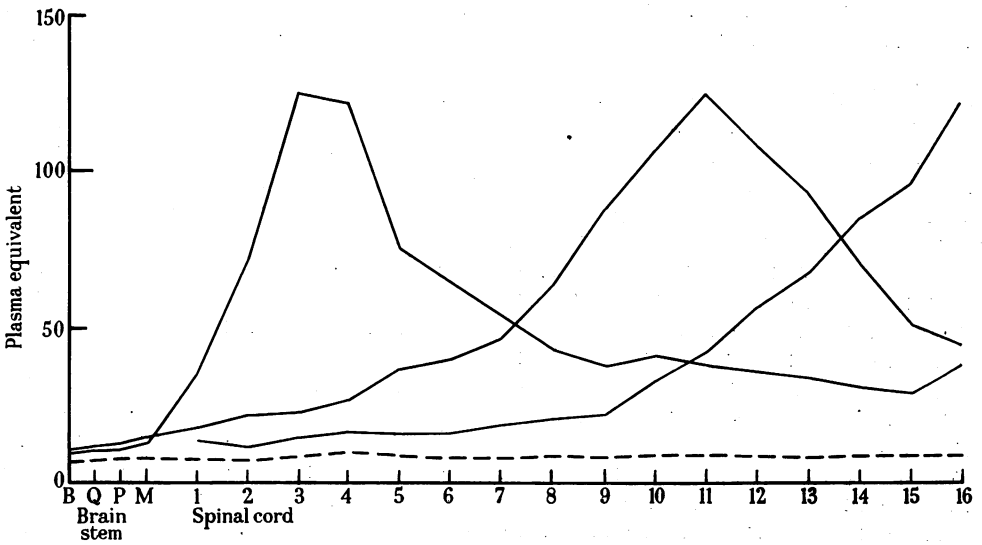

FIG. 5.—Composite diagram to show the mean radioactivities of the cerebrospinal axis of control rabbits (interrupted line) and of three groups of rabbits in which myelitis had been produced by inoculations of virus into the median, first lumbar spinal and sciatic nerves (continuous lines).

observed closely resembled those described by Levaditi (1926) for rabbits with herpetic encephalitis and myelitis. A noteworthy feature of herpes myelitis, however, is the variability in the histology of the lesions. Though the morphology of the predominant lesion was usually the same throughout any particular spinal cord, differing only in severity at different levels, there was often surprising variation between cords taken from animals which had presented essentially the same clinical disturbances. Flexner and Amoss (1925) referred to this histological variability, and noted a " wide disparity of lesions and no close relationship between their intensity and the severity of the symptoms ".

In the present study there was a broad correlation between the height of the radioactivity, expressed in plasma equivalents, and the severity of the morphological changes. In those portions in which the plasma equivalent had been substantially raised—to seven or more times the control value—the lesions were of the advanced form characterised by focal haemorrhages, destruction of neurones, infiltration of cord substance and meninges with many polymorphonuclear, macrophage and plasma cells, vascular cuffing and occasional areas of necrosis. In portions with lower plasma equivalents—roughly twice those of the controls—the morphological signs of injury were slight or even absent. Typically, these milder lesions assumed the form of congestion of the blood vessels, activation of the microglia, and accumulations of macrophages in perivascular spaces and beneath the meninges. At this stage there was usually no involvement of the grey matter nor evidence of damage to neurones. When the plasma equivalent lay between these values, the histological changes were of intermediate severity.

DISCUSSION

It has long been recognised that early in the acute inflammatory reaction, the local capillary endothelium becomes abnormally permeable to plasma proteins and other circulating substances of large molecular size. This modification in the walls of the smaller blood vessels has been found notably useful for locating sites of incipient inflammation in the skin and other organs. In the central nervous system, such raised vascular permeability proves particularly valuable for demonstrating foci of tissue injury by reason of two distinctive features of this organ : first, the almost complete impenetrability of the normal blood-brain barrier for proteins ; and, second, its loose extensible structure and the high proportionate volume of its tissue fluids (Wallace and Brodie, 1939 ; Davson, 1955). Consequently, once the plasma proteins are no longer restrained from escaping into the extravascular tissue spaces, there is little obstacle to their progressive accumulation at sites of acute inflammation in the brain and spinal cord.

In the present experiments, segmental infections of the spinal cord at various levels have been brought about by the injection of a neurotropic strain of herpes virus into three widely separated major nerve trunks. As is well known from the studies of Levaditi (1926) and others, such inoculations are followed within a few days by nervous disturbances which are first recognisable in those structures that are innervated from the spinal cord segment primarily involved, but which soon become widespread and end in the death of the animal. By the injection of radio-iodinated homologous plasma proteins into the general circulation followed by their localised escape thence into the extravascular tissue fluid spaces of the spinal cord, it becomes possible to identify the site of the major injury and to

determine quantitatively the gravity of the damage at all levels of the cerebrospinal axis. In all the animals examined in the present experiments, it was found that the segmental level corresponding to the particular nerve trunk inoculated coincided closely with the level for the peak of the radioactivity, and that from this maximum the latter fell roughly exponentially. This gradation was particularly noteworthy after inoculation of the first lumbar nerve, for with this trunk, the radioactivity of the various portions of the spinal cord was found to be distributed almost symmetrically about the highest value in the first lumbar segment.

It is of interest in connexion with the possible modes of spread of neurotropic viruses in the central nervous system that the distribution of the radioactivity in spinal cords infected at a particular level appears to bear little relationship to the intraspinal architecture of the incoming axons of the posterior spinal nerve roots. Yee and Corbin (1939) traced the centripetal pathways followed by these sensory axons for the first five cervical nerves in rabbits by determining the distribution of the degenerating fibres in the substance of the cord resulting from the surgical division of the individual posterior roots proximal to the spinal ganglion. In no instance did the signs of degeneration extend caudally for more than four segments, which in this region of the cord occupy a length of about 3 cm. Since the median nerve arises mainly from the sixth cervical segment, it is unlikely that its sensory descending collateral axons pass beyond the segment of the spinal cord included in portion five in our experiments. The marked elevation of the radioactivity of portions much caudal to number five, however, shows that the zone of inflammation has extended to segments much below the lowest extremity of the descending sensory collateral axons. It seems noteworthy, moreover, that whereas the intraspinal divisions of the sensory neurones of the median nerve pass mainly upwards, the signs of inflammation in the cord are more extensive in the segments caudal to the level of its primary involvement. The results of the injection of virus into the first lumbar spinal nerve also display a similar lack of agreement between the intraspinal architecture of the incoming sensory axons and the distribution of the elevated radioactivity in the infected cord. It would seem, therefore, that this virus, possibly because of its pantropic properties, becomes dispersed from its level of entry into the spinal cord through its highly orientated tissue spaces rather than intracellularly along axonal pathways.

SUMMARY

Herpes myelitis has been produced at various levels in the spinal cord of rabbits by the injection of a neurotropic strain of the virus into the median, first lumbar spinal or sciatic nerves.

Through the subsequent intravenous injection of ^{131}I-labelled homologous plasma proteins and their preferential accumulation at sites of inflammation, the extent and severity of the resulting myelitis at various segmental levels has been determined.

From the comparative segmental distribution of the radioactivity in the infected spinal cord, it appears that the spread of the virus from its level of entry is better explicable on the basis of dispersion through tissue fluids than along axonal pathways.

The method here employed has provided a better quantitative estimate of the severity of the myelitis caused by this virus than that obtained from histological

studies. It might also prove useful in experiments designed to disclose the initial sites of entry and subsequent dispersion of certain other neurotropic viruses in the central nervous system.

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REFERENCES

DAVSON, H.—(1955) *J. Physiol.*, **129**, 11.
FABER, H. K.—(1936–37) *Proc. Soc. exp. Biol., N.Y.*, **35**, 10.
FIELD, E. J.—(1952) *J. Path. Bact.*, **64**, 1.
FLEXNER, S. AND AMOSS, H. L.—(1925) *J. exp. Med.*, **41**, 215.
GOODPASTURE, E. W. AND TEAGUE, O.—(1923–24) *J. med. Res.*, **44**, 139.
GUTMANN, E.—(1942) *J. Neurol. Psychiat.*, **5**, 81.
LEVADITI, C.—(1926) 'L'Herpes et le Zona.' Paris (Masson).
MCCLELLAN, R. H. AND GOODPASTURE, E. W.—(1923–24) *J. med. Res.*, **44**, 201.
MARINESCO, G. AND DRAGANESCO, S.—(1923) *Ann. Inst. Pasteur*, **37**, 753.
SACHS, R. A. AND LUMMIS, W. L.—(1955) *Proc. Soc. exp. Biol., N.Y.*, **90**, 565.
VEALL, N. AND BAPTISTA, A. N.—(1954) *Brit. J. Radiol.*, **27**, 198.
Idem, PEARSON, J. D. AND HANLEY, T.—(1955) *Ibid.*, **28**, 633.
WALLACE, G. B. AND BRODIE, B. B.—(1939) *J. Pharmacol.*, **65**, 220.
YEE, J. AND CORBIN, K. B.—(1939) *J. Comp. Neurol.*, **70**, 297.