The RISA data, as presented in Figure 2, tend to substantiate our thesis that the inflammatory response in the intrapleural edema test is not produced immediately upon injection of the dye solution but develops gradually. However, at the end of the fifth hour, an inflammatory response had developed fully as indicated by the elevated ratio of relative RISA concentration. This raised the intrapleural colloid osmotic pressure and a new diffusion equilibrium between the pleural cavity and the pleural vasculature then had to be established.

A leveling off of the volume and protein concentration of the pleural fluid would indicate establishment of a new equilibrium. The data for 6 h post dye injection show that the leveling off process had begun. It is significant that the concentration of RISA/ml pleural fluid had become almost equal to that in the blood (Table II) – a ratio of 1 would denote this. This suggests that almost complete mixing of plasma protein with interstitial protein had occurred[10].

Zusammenfassung. Untersuchungen der auffallendsten Veränderungen des pleuralen Exsudates nach experimentellem Pleuralödem bei der Ratte sowie über die Ausbreitung des RISA aus den Pleuralgefässen in die Intercostalhautflüssigkeit sprechen dafür, dass sich die entzündliche Reaktion besonders langsam entwickelt, da sie selbst 5 h nach Farbinjektion noch nicht sichtbar wurde.

R. C. KELSEY[11] and G. J. FRISHMUTH

Biochemistry Department, Smith Kline and French Laboratories, Philadelphia (Pennsylvania, USA), 11 September 1967.

[10] We wish to thank Dr. M. FREE and J. PAULS of the Statistical Section for their advice and assistance in computing the statistical significance of the data. We wish to acknowledge the technical skill of J. BELZ in making the suction apparatus shown in Figure 1.

[11] Present address: Department of Biological Sciences, Drexel Institute of Technology, Philadelphia (Pennsylvania 19104, USA).

Lingually Induced Inhibition of Masseteric Motoneurones

The principal or main sensory nucleus of V is a compact nucleus of predominantly small cells which receives fine ascending branches of primary trigeminal afferents[1,2]. At its rostral extent it is bordered medially by the large cells of the trigeminal motor nucleus. In this area an extension of loosely arranged neurones from the main sensory nucleus caps the motor nucleus dorsally[1,3,4]. Some authors consider these cells to constitute a separate nucleus (e.g., the supratrigeminal nucleus of LORENTE DE NÓ)[1,4,5], while others describe them as the medial portion of the principal sensory nucleus[6]. Somatotopic analyses of single unit activity have shown that the buccal cavity is represented in this area[6,7]. It has been suggested on cytoarchitectural[4] and physiological[8] grounds that this region may contain an aggregation of interneurones active in trigeminal reflex pathways. To test this hypothesis we used the trigeminal reflex first described by MILLER and SHERRINGTON[3], in which lingual nerve stimulation produces reflex opening of the jaw in decerebrate cats.

A concentric bipolar stimulating electrode was inserted into the mesencephalic nucleus of V following cerebellectomy in the decerebrate, C_2 spinal sectioned cat. Animals were maintained under flaxedil and artificially respired. The ipsilateral masseteric nerve was dissected and cut distally; its central stump was placed on a bipolar silver wire electrode. This arrangement could be used either for recording the masseteric monosynaptic reflex induced by stimulation of the mesencephalic nucleus[9], or for the antidromic excitation of masseteric motoneurones in order to identify them during intracellular recording. The lingual nerve was sectioned bilaterally at the periphery and the central stumps were stimulated with a bipolar silver wire collar electrode. KCl or K-citrate filled micropipettes were used to record extracellular and intracellular potentials.

When low threshold afferent fibers in the ipsilateral lingual nerve were stimulated by a single pulse, a response in the form of a series of membrane potential changes were observed in masseteric motoneurones. A pulse of 10 μsec and 0.5–1.0 V could usually elicit this response, which was characterized by 3 phases: phase 1, hyperpolarization beginning with a latency of 2.3–2.9 msec with a peak at 8–10 msec; phase 2, depolarization with a peak at about 20 msec; phase 3, hyperpolarization peaking at about 40 msec followed by a gradual return to the prestimulus level (Figure 1A). These membrane potential changes corresponded closely to the changes in size of the masseteric reflex when tested following a conditioning lingual nerve stimulus (Figure 1B). The average size of the hyperpolarization in phase 1 was 5 mV. Antidromic and orthodromic spikes were readily blocked, and the membrane conductance was observed to increase during this phase. When recorded with KCl electrodes this hyperpolarization changed into a depolarizing potential with a similar time course to the original hyperpolarization. The evidence, thus, indicates that the hyperpolarization in phase 1 is an IPSP.

The degree of the return towards the control resting potential during phase 2 varied from cell to cell but in

[1] K. A. ASTRÖM, Acta physiol. scand. *29*, Suppl. *106*, 209 (1953).
[2] S. RAMÓN y CAJAL, *Histologie du Système nerveux de l'homme et des Vertébrés* (Consejo Superior de Investigaciones Cientificas, Madrid 1955), vol. 1, p. 839.
[3] F. R. MILLER and C. S. SHERRINGTON, Q. Jl exp. Physiol. *9*, 147 (1915).
[4] A. TORVIK, J. comp. Neurol. *106*, 51 (1956).
[5] R. LORENTE DE NÓ, in *Libro en honor Ramón y Cajal* (Jiminez y Molina, Madrid 1922), vol. 2, p. 13.
[6] L. KRUGER and F. MICHELE, Archs oral. Biol. *7*, 491 (1962).
[7] J. EISENMAN, S. LANDGREN and D. NOVIN, Acta physiol. scand. *59*, Suppl. 214, 1 (1963).
[8] C. R. JERGE, J. Neurophysiol. *26*, 393 (1963).
[9] A. HUGELIN and M. BONVALLET, J. Physiol. *49*, 210 (1957).

some cases the depolarization was sufficient to reach threshold and trigger a spike. It was observed that contralateral lingual stimulation induced similar triphasic effects on masseteric motoneurones and on the masseteric reflex. A discrete longitudinal midline section at the level of the motor nucleus abolished phase 1 of contralateral stimulation. This resulted in a 4–5 msec decrease in the peak latency of phase 2 and an increase in the peak size of the reflex to 130–140%. These results lead us to believe that phase 2 is due to an active excitatory process and is not a post-inhibitory rebound.

The amplitude of the hyperpolarization of the third phase was usually slightly less than that of the first. Monosynaptic and antidromic spikes were also blocked in this phase but the hyperpolarizing potential did not reverse with KCl electrodes as it did during the first phase. At a stimulation frequency of 5/sec the hyperpolarization of phase 3 was completely abolished with little change in that of phase 1, and transections of the brain stem about 2 mm caudal to the inferior limit of the motor nucleus completely abolished phase 3 and showed only a small reduction in phase 1. The results suggest that the inhibition of phase 3 is mediated by a polysynaptic path with some cells of the chain located caudal to the motor nucleus of V.

When recording in the supratrigeminal nuclear region an extracellular field potential was evoked from lingual nerve stimulation (Figure 2A, D). The maximum potential amplitude was recorded at about 500 μ dorsal to the motor nucleus. The first deflection of the lingually induced field potential was positive with a peak at 1.0 to 1.3 msec following the stimulus. This potential was considered to be due to the conduction of impulses in primary afferent fibers; it followed frequencies at least up to 300/sec with little change in the waveform and was similar to the 'tract wave' recorded by PORTER in the spinal trigeminal nucleus to lingual nerve stimulation[10]. In the experiment shown in Figure 2A, D the peak of the positive deflection occurred 1.3 msec after the stimulus and about 0.5 msec later a large negative potential began to develop. In every case single units could be isolated from this field potential. In a few instances we recorded EPSPs and spikes intracellularly in these units. The initial spike in this experiment began at about 1.7 msec latency (Figure 2B, E). Threshold stimulation induced 1 or 2 spikes but the maximum response (usually 5–8 spikes) could be reached at 1.5–2.0 times threshold. The frequency of the first 2 or 3 spikes was sometimes as high as 700 c/sec, with the later spikes discharging at lower frequencies (usually 500 c/sec). The incoming afferent volley arrived at the sensory nucleus at 1.3 msec and the rise phase of the first spike of the interneurone began at 1.7 msec. These latencies allowed time (0.4/msec) for only one synapse indicating that the interneurones are monosynaptically activated by lingual nerve stimulation.

Intracellular recording from masseteric motoneurones in the same animal showed that the initial hyperpolarization induced by lingual nerve stimulation had a latency of approximately 2.5 msec (Figure 2C, F). The rise phase of the first spike of the interneurone in Figure 2 began at 1.7 msec and the IPSP began at about 2.5 msec. This again allowed time for only one synapse, considering the time for spike development, conduction to the motoneurone, and synaptic delay. The total time between the arrival of the primary afferent volley at the sensory nucleus and the beginning of the IPSP was 1.2 msec. The division of the period into delays for one synapse between the primary afferent fiber and the interneurone, and one synapse between the interneurone and motoneurone, plus conduction time to the motoneurone is consistent with the observed results. The latency relationships shown in Figure 2 between the field potential, interneurone, and IPSP were consistent in 6 preparations.

The evidence presented here leads to the conclusion that stimulation of low threshold lingual nerve afferent fibers had the following effects on masseteric motoneurones: (a) induced short latency IPSP's via a disynaptic path, the internuncial neurones of which are

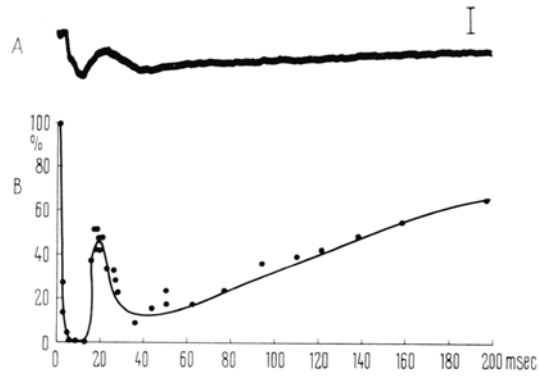

Fig. 1. Effect of a single shock to the ipsilateral lingual nerve on the membrane potential of a masseteric motoneurone (A) and on the masseteric reflex (B). A and B were recorded in the same cat. (A) resting potential: — 55 mV, positivity upward. (B) ordinate: reflex amplitude in % of control; abscissa: time interval between conditioning (lingual nerve) and test (mescencephalic nucleus of V) stimulation. Time scale for A and B is the same. Voltage calibration in A is 3 mV.

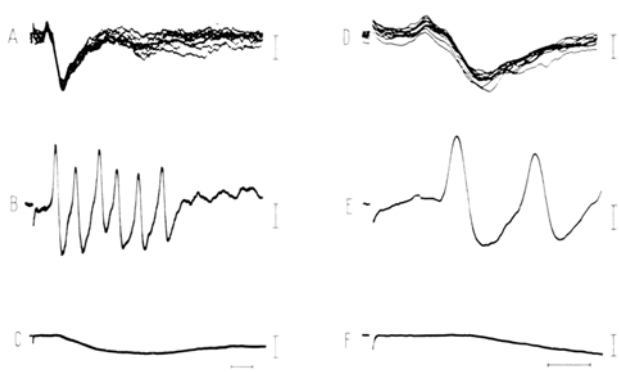

Fig. 2. Responses to single shocks delivered to the ipsilateral lingual nerve. A and D: 10 superimposed records, at stimulus frequency of 1/sec, of a field potential recorded from the same point in the supratrigeminal nuclear region. B and E: single extracellular records from a unit in the same region as A and D. C and F: intracellular records from a masseteric motoneurone, resting potential: — 65 mV (F is a photographic enlargement of C). The vertical bar for A and D represents 200 μV, for B and E 500 μV, for C 9 mV, and for F 5 mV. The time scale in C represents 2 msec and applies to A, B and C: that in F is 1 msec and applies to D, E and F. Positivity is upward in all records. All records are from the same preparation.

[10] R. PORTER, J. Physiol. *190*, 611 (1967).

located in the supratrigeminal nuclear region; (b) induced a phase of excitation that was partially obscured by the early inhibition; (c) induced a long latency inhibition via a polysynaptic path which extended caudal to the motor nucleus of V[11].

Zusammenfassung. Elektrische Reizung afferenter Fasern niedriger Reizschwelle im Nervus lingualis induzierte in den motorischen Neuronen des Nervus massetericus: (1) IPSPs von kurzer Latenz mittels einer bisynaptischen Bahn, deren Zwischenneurone im Gebiet der supratrigeminalen Nuclei liegen; (2) eine Erregungsphase, die teilweise von einer früh auftretenden Hemmung verdeckt ist; (3) eine Hemmung von langer Latenz mittels einer polysynaptischen Bahn, die sich kaudalwärts vom motorischen Ursprungskern des Nervus trigeminus erstreckt.

L. J. Goldberg and Y. Nakamura

Department of Anatomy and the Brain Research Institute, University of California, Los Angeles (California 90024, USA), 6 November 1967.

[11] This research was supported by a grant from the USPHS (No. MH-10083). Dr. Goldberg is a NIDR Postdoctoral Fellow. Dr. Nakamura is a Research Fellow from the Department of Neurophysiology, Institute of Brain Research, University of Tokyo (Japan).

Propagation of Mouse Hepatitis Virus (MHV-3) in Monolayer Cell Cultures from Liver of New-Born Mice

Mouse hepatitis virus (MHV-3) has been propagated in vitro with different techniques by several authors[1-4] and, particularly, in the cells derived from liver explants of mice fetuses and cultivated on reconstituted rat-tail collagen[5]. With this technique the virus replication was followed by an evident cytopathic effect.

This method is, nevertheless, very complicated and the cell growth is very slow. On the other hand, it is known that the liver cells obtained from fetuses or from new-born animals do not survive long when cultivated on a plain glass surface.

In order to gather further information on MHV-3 replication in vitro, it seemed of interest to test the possibility of replication of this virus on primary monolayer liver cell cultures obtained from new-born mice with a simple technique.

Materials and methods. Cell culture: The livers of new-born mice (Swiss strain) were pooled, finely minced with scissors, twice washed in cold Hanks' BSS, placed in 0.25% trypsin (Difco) and 0.20% methylcellulose (Fischer) in calcium- and magnesium-free Hanks' BSS, and agitated on a magnetic stirrer for 20–30 min at room temperature. The resulting suspension was centrifuged at 300 *g* for 10 min, decanted, and the sediment resuspended in the nutrient medium. The nutrient medium consisted of 20% of fresh inactivated calf serum, 0,5% of chick embryo extract and 0.1% of yeast extract (Difco) in Eagle's basal medium (Difco). The cells were counted, diluted in the nutrient medium to a concentration of 1×10^6/ml, distributed in 2 ml volumes in stationary tubes or in Leighton tubes containing a coverslip for the morphological observations, and placed in a 37 °C incubator. After 7–10 days a monolayer of polygon-shaped cells was formed. Subcultures were performed approximately every 10 days, using the same method described above.

These cells were passed through 9 subcultures; after this time degeneration of the cultures occurred.

Virus: MHV-3 was supplied by the American Type Culture Collection and maintained in our laboratory in receptive albino mice. The LD_{50} was determined according to Reed and Muench by using 3-week-old albino mice (Swiss strain) weighing about 10 g.

A 10^{-3} dilution of liver homogenates from moribund animals in Eagle's basal medium was made extemporaneously and centrifuged at 3000 rpm for 10 min.

For culture infection, the nutrient medium was eliminated and 2 ml of fresh nutrient medium containing 0.1 ml of viral suspension were placed in the tubes. The cultures were maintained at room temperature for 60 min. The virus suspension was then eliminated, the cell cultures twice washed with Hanks' BSS and incubated at 37 °C with fresh growth medium.

After 0, 12, 24, 36, 48 and 72 h respectively, the culture medium from 10 infected tubes was pooled and 10-fold diluted in sterile broth.

The biological virus titration was carried out with 0.1 ml of each dilution inoculated i.p. into 10 albino mice. At the same time, the coverslips were extracted from the

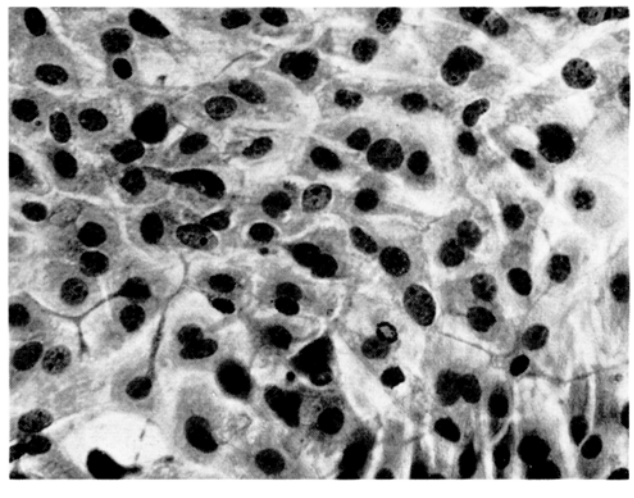

Fig. 1. Epithelial cell culture from trypsinized liver of new-born mice (8 days after trypsinization). Hematoxylin-eosin. × 250.

[1] R. F. Haff, Virology *18*, 507 (1962).
[2] T. Vainio and J. D. Judah, Expl molec. Path. *1*, 27 (1962).
[3] J. W. Hartley and W. P. Rowe, Proc. Soc. exp. Biol. Med. *113*, 403 (1963).
[4] L. Mallucci, Virology *25*, 30 (1965).
[5] F. Paradisi and F. Piccinino, G. Microbiol., in press.