4. Hayes, S. P., Dougherty, T. F., *Fed. Proc.*, 1952, v11, 67.
5. Michael, M., Whorton, C. M., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 754.
6. Schricker, R. L., Hanson, L. E., *Am. J. Vet. Res.*, 1961, v22, 580.
7. Christian, J. J., *Am. J. Physiol.*, 1955, v182, 292.
8. Vandenbergh, J. G., *Animal Behaviour*, 1960, v8, 13.
9. Davis, D. E., Read, C. P., PROC. SOC. EXP. BIOL. AND MED., 1958, v99, 269.
10. Treadwell, P. E., Rasmussen, A. F., *J. Immunol.*, 1961, v87, 492.
11. Eechaute, W., Demeester, G., LaCroix, E., Leusen, I., *Arch. Int. Pharmacodyn.*, 1962, v136, 161.
12. Barrett, A. M., Stockham, M. A., *J. Endocrinol.*, 1963, v26, 97.
13. Davis, D. E., Christian, J. J., PROC. SOC. EXP. BIOL. AND MED., 1957, v94, 728.
14. Hanks, J. H., *J. Immunol.*, 1935, v28, 95.

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## Rat Cerebral Cortical Oxygen Uptake Stimulating Factor from Sciatic Nerve.* (28884)

ROBERT J. DELLENBACK AND DAVID A. RINGLE† (Introduced by Shu Chien)
*Department of Physiology, College of Physicians and Surgeons, Columbia University, New York City*

A substance which stimulates oxygen consumption has been reported by Heller and Hesse(1) to be present in the sciatic nerve of the rat. This substance diffuses from the nerve during *in vitro* incubation in glucose-free medium at room temperature and is capable of increasing the rate of oxygen uptake of a fresh nerve subsequently tested in this medium. These authors(1) have named this material "activating" substance and the medium into which it has diffused "activating" medium.

The stimulation of oxygen uptake of rat sciatic nerve was observed with an "activating" medium prepared from the sciatic nerves of the rat. The experiments to be reported here were designed to test whether such a diffusible factor from nerve is capable of stimulating the oxygen uptake of rat cerebral cortical slices. In an attempt to determine the source of this "activating" substance the sciatic nerves were divested of their connective tissue sheaths (epineurium). The desheathed nerves and their sheaths were separately tested for effects on the oxygen uptake of cortex slices.

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† Present address: Midwest Research Inst., Kansas City, Mo.

*Methods.* I. *Manometry.* Manometers with a 1 mm bore, and 5 ml flasks were used with an American Instrument Co. circular Warburg apparatus. The temperature of the water bath was maintained at 37.34° ± 0.02°C and the flasks were shaken at 120 oscillations per minute. The rims of the center wells were greased with anhydrous lanolin and subsequently filled with 0.1 ml of 5% KOH. The surface area for absorption of $CO_2$ was increased by using a fluted filter paper. The flasks were gassed with pure oxygen for a period of 10 minutes, followed by a 10-minute temperature equilibration period, after which the manometers were closed. The first, or zero time, reading was taken 5 minutes later and subsequent readings were taken every 10 minutes for one hour.

II. *Solutions.* Two incubation media were used in these experiments. The millimolar quantities of their constituents are given in Table I. Fresh media for each experiment were prepared by pipetting measured quantities from stock solutions. The substrates for the Krebs III(2) medium were stored under refrigeration for no more than 4 days.

III. *Preparation of "activating" medium.* Three male Sherman rats were sacrificed by decapitation with a guillotine and their sciatic-peroneal nerves quickly dissected out.

TABLE I. Media Used in Incubation Procedures.

| | A Non-substrated phosphate saline(1) (mM) | B Krebs III (mM) |
|---|---|---|
| $Na^+$ | 153.5 | 141.4 |
| $K^+$ | 3.0 | 5.9 |
| $Ca^{++}$ | — | 2.5 |
| $Mg^{++}$ | 1.2 | 1.2 |
| $Cl^-$ | 125.0 | 127.8 (122.8)* |
| $SO_4^=$ | 1.2 | 1.2 |
| $HCO_3^-$ | | 3.6 |
| Phosphate | 17.5 | 3.5 |
| Pyruvate | | 4.9 |
| Fumarate (di-$Na^+$) | | 5.4 |
| Glutamate | | 4.9 |
| Glucose | | 11.5 |

* Chloride concentration used in these experiments due to omission of calcium from the solution.

Immediately following removal, a nerve was cleaned of adhering extraneous tissue and the epineurium carefully removed. The 6 desheathed nerves and the 6 sheaths were placed into separate beakers containing a predetermined volume of one of the above media. These beakers were covered and allowed to stand, with periodic gentle agitation, at room temperature for 2 hours. The fluids were then poured into conical centrifuge tubes and centrifuged at 2000 rpm for 10 minutes. The solutions were then immediately pipetted into Warburg flasks and the flasks iced prior to addition of the brain slices. The nerves and sheaths were drained of excess fluid, blotted on filter paper and immediately placed into tared weighing bottles for determination of their wet weights.

Control media received similar treatment except for the inclusion of tissue.

IV. *Preparation of cortical slices.* Male Sherman rats weighing 300-400 g were used throughout. The brains of 3 animals were used for each experiment. The heads were moved with the use of a guillotine and the brains quickly removed. The brains were placed in a covered Petri dish on a filter paper disc which had been previously moistened with the control medium to be used in the experiment. The Petri dish moist-chamber was chilled by placing it into an especially designed lucite box in which water had been previously frozen. The time for removal of all 3 brains was approximately 6 minutes.

Brains were cut into right and left halves by a mid-line incision. One of the halves was removed from the moist chamber, the pia carefully stripped away and 4 surface slices (0.4-0.5 mm thick) cut using a razor blade and template. Immediately after cutting, the slices were serially arranged on the moistened filter paper in the Petri dish to provide separate portions for each Warburg flask. Second slices from different animals, opposite hemispheres and different sites on the hemisphere were combined with the first slices. This procedure of using 2 slices per flask was used to reduce, as much as possible, effects due to individual brain variability and possible regional differences in oxygen uptake. The 4 slices from each hemisphere were routinely taken from the same surface regions and in the same order, to facilitate the combination of tissues into groups. The time required for preparation of all the brain slices used in any one experiment was approximately 20 minutes.

Grouped slices were weighed on a Roller-Smith torsion balance and placed into iced Warburg flasks. Total time from initial decapitation to the completed loading of 16 manometer vessels (12 experimental, 4 thermobarometers) was approximately 50 minutes.

V. *Analysis of results.* Regression equations were calculated using the data from the 10-, 20-, 30- and 40-minute readings disregarding the origin as a point. $QO_2$ values for any experimental series were then calculated from the regression equation by using 60 minutes as the time factor. The $QO_2$ values reported here are thus calculated, and not measured values. The significance between one series of experiments and another was calculated using the "Student" t test on the measured values obtained at 40 minutes.

*Results.* I. *Non-substrated phosphate saline medium.* A first series of experiments was performed to test whether either sciatic nerve or the sheath of sciatic nerve contains a substance which is capable of diffusing into the medium during an incubation period and is subsequently capable of stimulating the

TABLE II. Results of a Non-Activated Control and Nerve- and Sheath-Activated* Non-Substrated Phosphate Saline Solutions on Oxygen Uptake of Rat Cerebral Cortical Slices.

| Medium | No. of exp | Regression equation† | 40 min values‡ | $QO_2$§ |
|---|---|---|---|---|
| Control | 12 | $Y = .044X + .36$ (n=48) | $2.1 \pm .7$ | 3.0 |
| Nerve activated | 12 | $Y = .075X + .38$ (n=48) | $3.3 \pm .5$ ($P < .01$) | 4.9 |
| Sheath " | 12 | $Y = .062X + .39$ (n=48) | $2.8 \pm .7$ ($P < .015$) | 4.1 |

* 2-hr room temperature (24-26°C) incubation.
† Values in parentheses represent total number of determinations from which regression equations have been calculated.
‡ Values are means ± S.D.
§ $\mu$l $O_2$ consumed/mg dry tissue/hr. Dry weights (16% of fresh wt) were determined on a large series of cortical slices by drying at 100°C for 72 hr.

oxygen uptake of cerebral cortical slices. The "activating" medium will be referred to as "nerve-activated" or "sheath-activated," depending on the source of the activating substance.

The results of these experiments using medium A (Table I) for incubation are presented in Table II. The $QO_2$ for cerebral cortical slices in nerve-activated medium averaged 4.9 as compared with a control of 3.0. The average 40-minute values (± S.D.) listed in Table II show a statistically significant difference ($p<0.01$) between these groups. The results in Table II further show a sheath-activated $QO_2$ value of 4.1 which is significantly greater than the control ($p< 0.015$). The difference between the two stimulated $QO_2$ values can be partially accounted for on the basis of the wet weight/incubation volume ratio which averaged 26.5 mg/ml for nerve and 18.8 for sheath.

II. *Calcium-free Krebs solution.* Medium A used for the above series contained no substrate. To study whether the presence of a substrate is important in the release of the activating substance, use was made of a well-substrated Krebs medium (Table I).

Removal of calcium from substrated Krebs III medium is known to stimulate *in vitro* respiration of rat brain slices(2). Calcium was therefore omitted from this solution to make it more comparable to the non-substrated phosphate saline solution and, therefore, to make the substrates limiting.

The results of a similar series of experiments, but using Krebs III medium lacking calcium are presented in Table III. The cerebral cortical $QO_2$ from a control medium in which no tissue had been incubated was 23.1. The results from the nerve and sheath activated solutions show $QO_2$ values of 23.3 and 22.5 respectively, which are not significantly different from the controls ($p>0.50$; $p> 0.10$) at the 40-minute point. The average wet weight/incubation volume ratio for nerves was 28.6 mg/ml and for sheath 21.0 mg/ml.

*Discussion.* Although available data on $QO_2$ of rat brain slices incubated in calcium-free media are limited, $QO_2$'s observed in the present studies compare favorably with those reported by Elliott & Bilodeau using calcium-free unsubstrated media(3) and Krebs using calcium-free substrated media(2).

TABLE III. Results of a Non-Activated Control, Nerve-Activated and Sheath-Activated* Krebs III Solutions Lacking Calcium on the Oxygen Uptake of Rat Cerebral Cortical Slices.

| Medium | No. of exp | Regression equation† | 40 min values‡ | $QO_2$§ |
|---|---|---|---|---|
| Control | 12 | $Y = .373X + .69$ (n=48) | $15.5 \pm .8$ | 23.1 |
| Nerve activated | 12 | $Y = .378X + .60$ (n=48) | $15.6 \pm 1.3$ ($P > .50$) | 23.3 |
| Sheath " | 12 | $Y = .366X + .50$ (n=48) | $15.0 \pm .9$ ($P > .10$) | 22.5 |

*†‡§ See legend of Table II.

A stimulation of the respiration of rat cerebral cortical slices *in vitro* by a medium in which rat sciatic nerve had been previously incubated has been shown by the above experiments. These results extend the earlier observations of Heller and Hesse(1) to show that the oxygen uptake of cerebral cortical slices, as well as of nerve, are stimulated by some factor which diffuses from sciatic nerve. A stimulation was observed if the medium in which the nerve was incubated contained no added substrates, but was absent if the incubation medium was well substrated. The factor increases the respiration of brain slices by a small amount compared with the marked stimulation produced by the presence of added substrates (Tables II and III). Since sheaths of nerves, incubated in the same manner as nerves, are capable of producing a similar stimulation, nerve is not a unique source of the "activating" substance.

The nature of the activating factor is unknown. Diffusible substances, as yet unidentified, have been recently shown to be released by peripheral nervous tissue during an incubation period(5). The nature of and the role played by these diffusible "activating" substances from peripheral nerve require further investigation.

*Summary.* Stimulation of the *in vitro* oxygen uptake of rat cerebral cortical slices by some factor which diffuses from rat sciatic nerve or the sheath of sciatic nerve during a 2-hour incubation period at room temperature has been shown. The incubation medium used was phosphate buffered, possessing the normal ionic constituents except for calcium and lacking exogenous substrates. The control $QO_2$ determined in this medium was 3.0 with values of 4.9 ($P<0.01$) and 4.1 ($P<0.015$) resulting from nerve- and sheath-activated media respectively. $QO_2$ values determined in a well-substrated medium (Krebs III solution lacking calcium) did not show any stimulation. The results of these experiments suggest that both the nerve and its sheath are good sources of an oxygen uptake stimulating material. The nature of the substance is unknown.

1. Heller, I. H., Hesse, S., *Science,* 1961, v133, 1708.
2. Krebs, H. A., *Biochim. et Biophys. Acta,* 1950, v4, 249.
3. Elliott, K. A. C., Bilodeau, F., *Biochem. J.,* 1962, v84, 421.
4. Heller, I. H., Wolfe, L. S., Hesse, S., *J. Neurochem.,* 1962, v9, 443.
5. Koranyi, L., Lissak, K., *Acta Physiol. Acad. Sci. Hung.,* 1962, v21, 65.

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## Cerebral Cortical Oxygen Uptake Stimulating Factor from Cerebral Cortical Slices.* (28885)

DAVID A. RINGLE† AND ROBERT J. DELLENBACK (Introduced by Shu Chien)
*Department of Physiology, College of Physicians and Surgeons, Columbia University, New York City*

A factor which diffuses from rat sciatic nerve during *in vitro* incubation in a substrate-free medium at room temperature has been reported(1). In the presence of this factor freshly excised nerves(1) and cerebral cortical slices(2,3) show a stimulation of their respiration. This material has been referred to as the "activating" substance (1,2,3). Sciatic nerve and its connective tissue sheath both appear to be a source of this material.

The present experiments were designed to test if an activating substance is also released from rat cerebral cortical slices during room temperature incubation which is subsequently capable of stimulating the respiration of cere-

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† Present address: Midwest Research Inst., Kansas City, Mo.