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Effect of Biotin on Chick Spinal Ganglia in Tissue Culture.*

AGNES S. BURT. (Introduced by Paul Weiss.)

From the Department of Zoology, The University of Chicago.

Hamilton and Plotz[1] have recently reported that biotin in concentrations of 1/6 to 5/6 γ per cc in tissue culture media markedly improves growth of embryonic chick and mouse explants, particularly those containing nerve cells. As their work appears to have been done on embryo fragments containing several different types of tissue, and on brain, nerve growth from which is very difficult to measure because of the fineness of processes and the influence of the liquefaction which frequently occurs in such cultures,[2] their experiments have been repeated using isolated spinal ganglia of embryonic chicks with the purpose of appraising biotin as a possible stimulant of axon regeneration. Spinal ganglia, previously used as test material in this laboratory,[3] produce abundant growth of axons, easily demonstrable in fixed material by silver stains, as well as of spindle cells and macrophages.

One hundred and eight spinal ganglia from the lumbo-sacral and thoracic regions of 9- to 13-day-old embryos were explanted into chicken plasma and embryo extract. In 55 cases, Biotin Concentrate No. 1000 (S. M. A. Corporation), furnished in 50% ethanol, was added to the clot in modified Tyrode's solution so that the final amount of biotin added to the medium was 1/3 γ per cc (the optimum stimulating concentration according to Hamilton and Plotz); and in 53 control cases, Tyrode's solution containing a volume of 50% alcohol equivalent to the volume of the biotin concentrate was added. The pH of the Tyrode's solution was adjusted to 7.4 with $NaHCO_3$ before it was added to the cultures in all cases. Ganglia from one side of any given embryo were used for controls and those from the other side for biotin treatment, but no attempt was made to pair cultures because individual differences among ganglia due to treatment during removal and, apparently, to inherent factors preclude the use of this method of comparison.

Observations were made on the living cultures at 24-hour intervals, and all cultures were fixed after 2 days, as previous experience has shown that maximum axon growth is obtained with this technic on the second day of incubation and that regression of nerve fibers begins soon after. Preparations were stained by Bodian's protargol method and density of nerve fibers, spindle cells, and macrophages was recorded by an arbitrary system in which Class I signified very sparse growth, Class II moderate growth, Class III dense growth, and Class IV very dense, almost solid growth. Standards of nerve fiber density have been illustrated in a previous publication.[3]

The 108 cultures were explanted in 5 groups, each containing control and treated cultures. In only one of these groups the biotin-treated cultures grew better than the controls, while in the other 4 groups biotin treatment either had no effect on growth of the various cell components or appeared to be slightly inhibitory. Results of all 5 experiments are summarized in Table I, which shows the total number of cultures falling in each arbitrary density class for each of the cell types studied.

As may be seen from this table, no statistically significant difference between the control and biotin-treated cultures was revealed

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1 Hamilton, H. L., and Plotz, H., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 133.

2 Weiss, P., *Anat. Rec.*, 1934, **58**, 299.

3 Burt, A. S., *J. Cell. and Comp. Physiol.*, 1943, **21**, 145.

TABLE I.
Effect of 1/3 γ Biotin/cc Medium on Growth of Spinal Ganglia in Plasma.

Density class	Nerve fibers			Spindle cells			Macrophages		
	Control	Biotin	X^2	Control	Biotin	X^2	Control	Biotin	X^2
I	1	3	1.00	6	9	0.27	4	8	1.34
II	20	17	0.43	20	28	1.34	25	23	0.08
III	20	22	0.10	25	16	2.44	16	15	0.13
IV	12	13	0.00	2	2	0.00	8	9	0.00
Total	53	55	1.53	53	55	4.05	53	55	1.55
P between	0.95—0.50			0.20—0.10			0.95—0.50		

by use of the Chi Square method of analysis. The radius of cell outgrowth from each ganglion was measured with an ocular micrometer; and the average outgrowth from the controls was found to be 650 μ as compared with 602 μ for the biotin-treated ganglia. However, because of the large standard deviations involved, this difference is not significant.

As a further check, 72 cultures of spinal ganglia were made in a liquid medium consisting of 5 parts Tyrode's solution plus 1 part chicken serum, 1/3 γ biotin per cc being added to half the cultures and 50% alcohol equivalent to the volume of the biotin concentrate to the controls. In this medium, biotin appeared to have a pronounced inhibitory effect on all classes of cells. Nearly half of the biotin cultures showed no trace of growth, while the control ganglia produced many axons and a moderate number of spindle cells and macrophages.

Brain, though less adequate for tests of this kind, was examined in 10 cultures of 6-day embryonic chicks to which biotin was added and in 10 controls to which no vitamin was added. A narrow ring of mobilized nerve cells, such as was ascribed by Hamilton and Plotz to amitotic division, was present around most of the explants in biotin, and around most of the control cultures as well. Such a neuroblastic fringe is well known to be a passive result of plasma retraction in liquefying brain cultures[4,2] and cannot be considered evidence of nerve cell proliferation.

In order to determine possible biotin effects on tissue other than nerve, 20 explants of 6-day embryonic chick heart were made, 10 in biotin-plasma and 10 controls in alcohol-plasma. After 3 days' incubation, the radius of the growth zone was 944 μ in the controls and only 774 μ in the biotin cultures. Because of the large standard deviation obtained, the difference was not significant.

Discussion. The presence of alcohol in all cultures, biotin as well as controls, has depressed growth in comparison to several hundred alcohol-free cultures made in this laboratory for other purposes. This inhibitory effect of about 0.2% alcohol *in vitro* contrasts with findings in living tadpoles where the threshold for axon injury is over 0.5% and sheath cells are frequently unaffected by as much as 3% alcohol.[5] Furthermore, our cultures showed greater inhibition by the same concentration of alcohol in liquid medium than in plasma clots. Grossfeld[6] has reported a similar increase of sensitivity of heart fibroblasts to extreme pH values in liquid media as compared to plasma.

The presence of biotin in our cultures definitely had no stimulating effect on cell and fiber growth in plasma and depressed growth significantly in liquid medium. The discrepancy between these results and those of Hamilton and Plotz might be explained by possible variations in the chemicals used or by the different standards used in assessing growth. Their paper fails to give details of the criteria used in rating nerve fiber growth; moreover, in repeating their procedure in 16 paired cultures of complex tissue from the midline of 6-day-old chick embryos, I found that

[4] Olivo, O. M., *Arch. f. exp. Zellforsch.*, 1927, **4**, 43.

[5] Speidel, C. C., *J. Comp. Neur.*, 1936, **64**, 77.

[6] Grossfeld, H., *Z. f. Zellforsch. u. mikr. Anat.*, 1936, **25**, 312.

whatever axon growth did occur was almost completely obscured by other cells.

In the rat, neither biotin deficiency[7] nor administration of amounts of the vitamin far in excess of normal intake[8] has been found to affect the rate of regeneration of crushed sciatic nerves significantly. These findings in the living animal in conjunction with those reported here in tissue culture do not support the contention that biotin is a stimulant of axon growth.

Summary. In a total of 236 tissue cultures, no evidence was found for a growth stimulating action of biotin on embryonic chick axons, neuroblasts, spindle cells, or macrophages. In view of the close resemblance between axon regeneration *in vivo* and *in vitro*,[9] these results discourage the view that biotin might be clinically useful as a stimulant of nerve regeneration, at least during the crucial outgrowth phase.

[7] Fischer, E., *Fed. Proc. Am. Soc. Exp. Biol.*, 1943, **2**, 13.

[8] Lazere, B., Thomson, J. D., and Hines, H. M., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 81.

[9] Weiss, P., *Growth*, 1941, **5**, 163.

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Depression of Experimental Polycythemia by Stomach U.S.P.; Presence of Choline in Stomach U.S.P.*

JOHN EMERSON DAVIS

From the Department of Physiology and Pharmacology, University of Arkansas School of Medicine, Little Rock.

It was reported that the daily administration of 5 g of Stomach U.S.P. (Ventriculin†) to polycythemic animals had no effect on the red blood cell count, although the feeding of raw liver produced a reduction of erythrocytes.[1] Subsequently, we showed[2] that choline chloride depressed experimental polycythemia in the same manner as raw liver, and later[3] the probable mechanism of action was reported.

The present communication reports the results of the administration of a higher (10 g) daily dose of Ventriculin to polycythemic dogs, and the proof that Ventriculin contains choline which is probably the substance responsible for the depression of polycythemia.

Procedure. Two normal and 2 splenectomized dogs were maintained on a constant adequate diet. Polycythemia was produced in 2 of the animals by the daily oral administration of cobalt chloride according to a method described previously,[4] and the other 2 dogs were made polycythemic by the daily injection of posterior pituitary solution.[5] After the development of the polycythemias, each dog was fed 10 g of Ventriculin daily in addition to the erythropoietic-stimulating substances.

To determine the possible existence of choline in Ventriculin, a chemical analysis was made by the method of Johnson, Irvine, and Walton.[6] In addition, a pharmacological test was made by acetylating a deproteinized aqueous extract of Ventriculin according to the method of Mentzer *et al.*[7] and determining its effect on the blood pressure of a dog.

Results. Fig. 1 shows the effect of the daily oral administration of 10 g of Ventriculin upon the red blood cell counts of 4 polycythemic dogs. It will be noted that this

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† Supplied by Parke, Davis & Co., Detroit, Mich.

[1] Davis, J. E., *Am. J. Physiol.*, 1938, **122**, 397.

[2] Davis, J. E., *Am. J. Physiol.*, 1939, **127**, 322.

[3] Davis, J. E., *J. Pharm. and Exp. Therap.*, 1940, **70**, 408.

[4] Davis, J. E., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 96.

[5] Davis, J. E., *Am. J. Physiol.*, 1942, **137**, 699.

[6] Johnson, C. G., Irvine, J. L., and Walton, C., *J. Biol. Chem.*, 1939, **131**, 425.

[7] Mentzer, C., Corteggiani, E., and Carayon-Gentil, A., *Bull. soc. chim. biol.*, 1939, **21**, 503.