h_0, then runs along the T_0-axis to $(\theta, 0, 0)$, and then has a final segment parallel to the final segment of h_0). It is easily seen that, given any $\alpha_1 < \alpha_2 < \ldots < \alpha_n < \omega_1$, then $D^*_{\alpha_1, \alpha_2, \ldots, \alpha_n, \omega_1}(h_0, h_\theta) = 0$ for $\theta > \alpha_n$. On the other hand, in any homeomorphism of the bases of h_0, h_θ, the points $(\omega_1, 0)$ of each base must correspond, whence $D_\alpha(h_0, h_\theta) = 1$, all $\alpha < \omega_1$. Thus h_0 is in the closure of the family (h_θ) with respect to U^* but not with respect to $\bar{U}$.

[1] A. J. Ward, "A Generalization of the Fréchet Distance of Two Curves," these PROCEEDINGS, **40**, 598, 1954. Reference should be made to this paper, referred to as "W," for the notation employed. The present paper, like W, was written while the author was in receipt of a Smith-Mundt grant.

[2] I.e., as in W.

[3] Cf. M. Morse, *Topological Methods in the Theory of Functions of a Complex Variable* ("Annals of Mathematics Studies," No. 15 [Princeton, 1947]), and W.

[4] In fact, $f\omega_1(x, y)$ will be zero for all points we actually use in E.

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*A NERVE GROWTH-STIMULATING FACTOR ISOLATED FROM SARCOMAS 37 AND 180**

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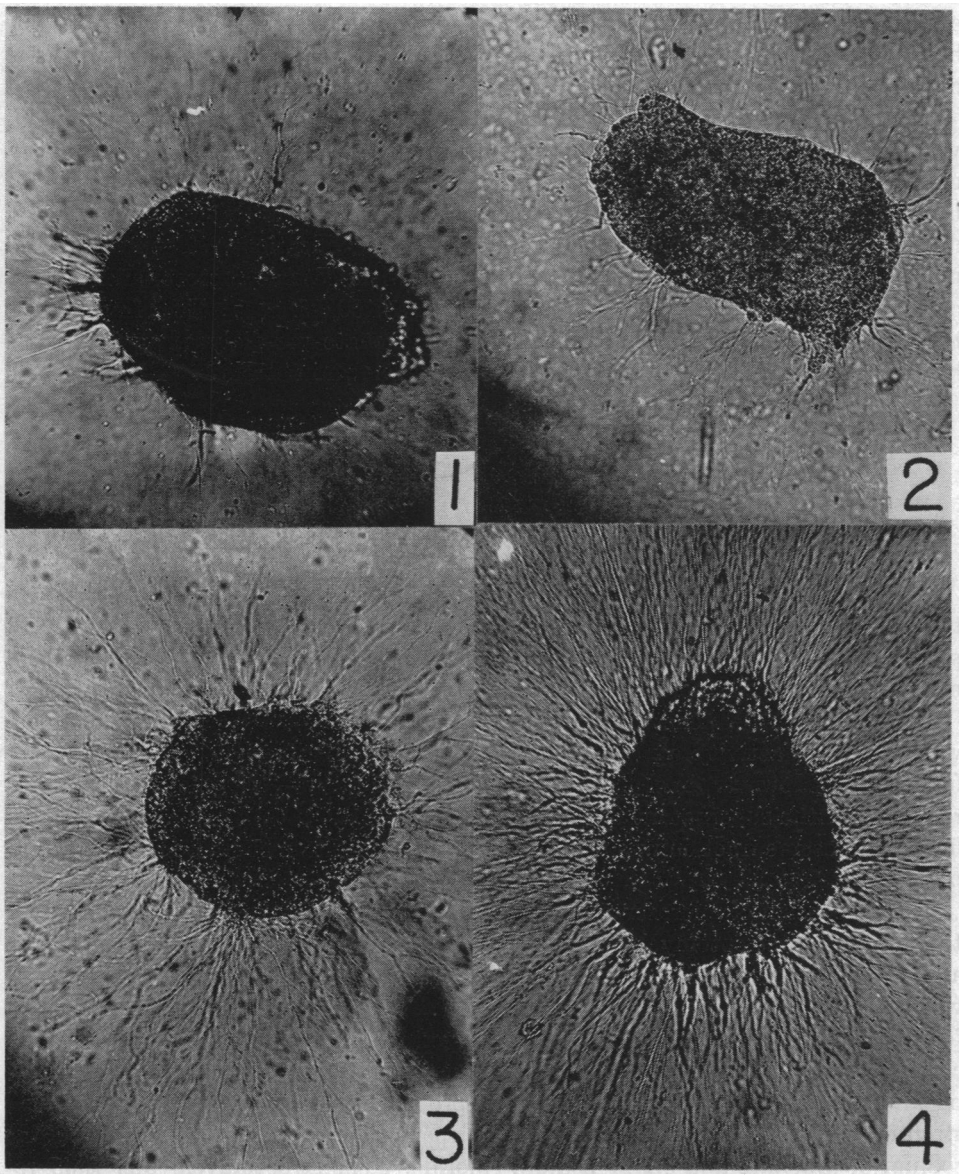
A growth-stimulating effect of mouse sarcomas 37 and 180 on the sensory and sympathetic ganglia of chick embryos has been reported.[1] The response of the ganglia to the transplantation of the tumor into 3- to 4-day chick embryos is characterized by a numerical hyperplasia, a cellular hypertrophy, acceleration of differentiation of the ganglia, and an atypical distribution of nerve fibers. The transplantation of the tumor onto the allantoic membrane[2] gave evidence for the humoral nature of the agent.

Explantation of the tumor in vitro in close proximity to sensory or sympathetic ganglia resulted in an exceptional outgrowth of nerve fibers.[3] The parallelism between the tumor effects in vivo and in vitro suggested that we are dealing in both instances with the same agent.

We have now found that cell-free homogenates of the tumors can duplicate in tissue culture the effect of the actively growing tumor. For the assay procedure, hanging-drop preparations were made containing $^1/_3$ plasma (rooster), $^1/_3$ chick extract (5 per cent extract of 10-day chick embryos), and $^1/_3$ of the material to be tested. Saline was used in the controls. Each culture contained a sympathetic ganglion isolated from a 10-day chick embryo. The cultures were observed after 18 hours of incubation at 37° C., and the growth of the fibers was semiquantitatively recorded from 1+ to 4+ (Pl. I, Figs. 1–4). The assay was sensitive to twofold changes in concentration of the active material; smaller changes were not detectable by gross observation of the ganglia.

Preliminary experiments had shown that extracts of sarcomas 37 and 180, which had been grown in the mouse, were almost completely inactive in stimulating the growth of nerve fibers, as were extracts of mouse liver and muscle. However,

PLATE I



FIGS. 1–4.—Microphotographs of living sympathetic ganglia after 18 hours of incubation, showing the nerve growth–promoting effect of increasing concentrations of the tumor extract. The chloroform-treated nucleoprotein fraction was used. Fig. 1, control; Fig. 2, 0.2 mg/ml (recorded as 1 plus); Fig. 3, 0.4 mg/ml (recorded as 2 plus); Fig. 4, 0.8 mg/ml (recorded as 4 plus).

after passage of the sarcomas through the chick embryo, extracts were invariably effective. The tumor was then routinely obtained by transplantation into the body wall of the 3-day chick embryo. The tumors were allowed to grow for 5–7 days before they were harvested.

The intracellular localization of the growth-promoting material was then investigated. Ten per cent homogenates of the tumor were prepared in isotonic sucrose (0.25 *M*, pH 7.4), according to the method of Schneider.[4] The procedure for the isolation of nuclear, mitochondrial, microsomal, and supernatant fractions by differential centrifugation was followed, with two modifications. The nuclear and mitochondrial fractions were washed four times with isotonic sucrose, and the microsomal fraction was isolated by centrifugation at 100,000 × g. Each fraction was then made up to the original volume of the homogenate and dialyzed against saline before assaying for its activity. The results are shown in Table 1. Practically all

TABLE 1

INTRACELLULAR LOCALIZATION OF NERVE GROWTH-PROMOTING FACTOR FROM SARCOMA 37 AFTER PASSAGE THROUGH CHICK

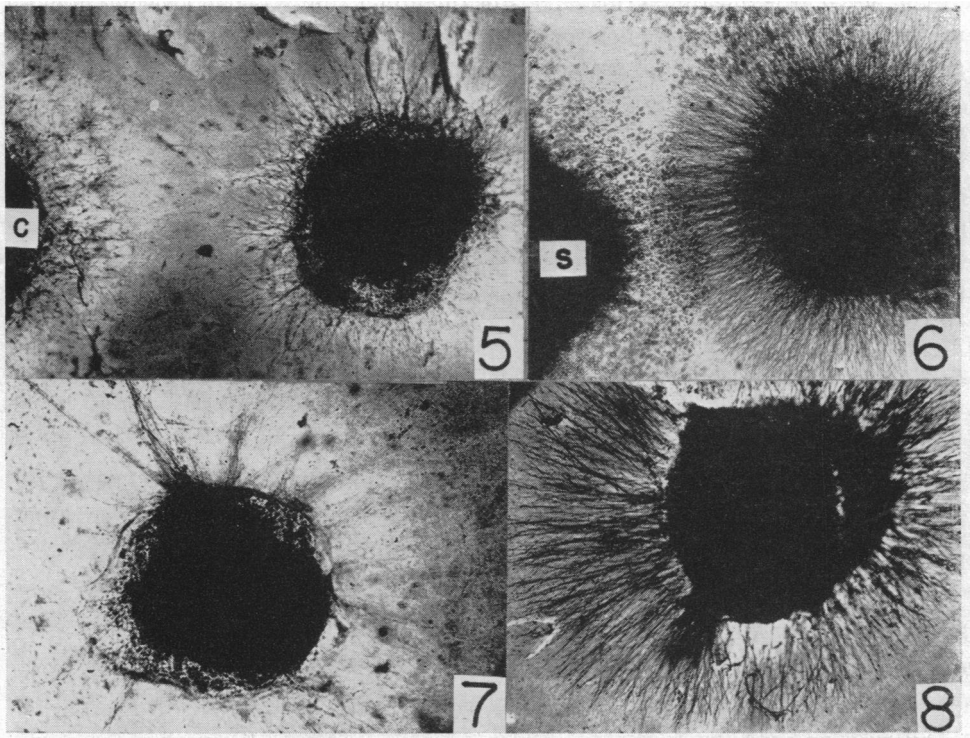
Cell Fraction Added	Growth of Nerve Fibers	Cell Fraction Added	Growth of Nerve Fibers
None	−	Mitochondria	±
Whole homogenate	++++	Microsomes	++++
Nuclei	−	Supernatant	−

the activity resided in the microsomal fraction, which contained approximately 16 per cent of the dry weight of the original tumor. In some instances the microsomal fraction showed a somewhat greater activity than the original homogenate.

The clear reddish pellet of microsomal material may be dispersed in distilled. water and the activity completely sedimented by centrifugation at 100,000 × g for 1 hour. However, in slightly alkaline solutions (pH 9–10) some of the activity remained in the supernatant fluid after centrifugation. At pH 5.6 all the activity in the microsome fraction was precipitated; the precipitate could be redispersed at pH 7.4.

The suspension of microsomes in distilled water could be further fractionated by the addition of streptomycin, which precipitates highly polymerized nucleic acids and nucleoproteins.[5] Streptomycin sulfate (from stock 0.2 *M* solution, pH 7.2) was added to a final concentration of 0.02 *M*. The mixture was allowed to stand for two hours in the cold and then centrifuged for 5 minutes at 8,500 × g. The clear reddish supernatant (containing home-proteins) showed no absorption peak at 260 mμ and no nerve growth–promoting activity. The precipitated streptomycin-nucleoprotein complex was dispersed in a solution containing 0.2 *M* sodium bicarbonate and 0.2 *M* sodium chloride. The streptomycin was then removed by dialysis for 24 hours against 0.2 *M* sodium bicarbonate and, finally, 24 hours against distilled water. This fraction, containing all the microsomal nucleic acid, possessed practically all the activity of the whole homogenate. The ratio of the absorption peak at 260 mμ to that at 280 mμ was 1.61.

PLATE II



FIGS. 5–8.—Microphotographs of silver-impregnated sensory ganglia, comparing the effect of the intact tumor with the growth-stimulating effect of the cell-free extract of the same time. Fig. 5, control lumbar ganglion of 7-day embryo combined with heart of check embryo (*C*); Fig. 6, ganglion combined with two fragments of sarcoma 37 (*S*); Fig. 7, control ganglion; Fig. 8, ganglion growing in a medium to which the cell-free extract of the tumor was added.

One volume of chloroform was added to 10 volumes of the nucleoprotein solution (in distilled water). After gentle mixing by inversion for 10 minutes, the resulting milky suspension was centrifuged at 8500 × g for 5 minutes, and the clear, somewhat opalescent, supernatant fluid was found to contain from 50 to 100 per cent of the original activity. The solution showed a typical nucleoprotein absorption curve, with a peak at 260 mμ and a 260 to 280 mμ absorption ratio of 1.78. The active material was heat-labile; the activity was completely destroyed by heating for 5 minutes at 80° C. The material was nondialyzable. When adjusted to pH 9–10, the activity no longer was sedimented in the ultracentrifuge (1 hour at 100,000 × g). This soluble active material could again be precipitated with streptomycin. Our purest preparation contained approximately 3 per cent of the dry weight of the original tumor. It was found to contain 66 per cent protein (as determined by the procedure of Lowry *et al.*,[6] using bovine albumin as a standard), 26 per cent ribosenucleic acid (determined by the oricinol procedure,[7] using adenosine as a standard) and less than 0.3 per cent desoxyribosenucleic acid (determined by the diphenylamine reaction,[7] using desoxyribose as a standard).

The nerve growth–stimulating effects of the intact tumor, when growing in close proximity to a sensory ganglion, are compared in Plate II, Figures 5–8, with the growth-stimulating effects of extracts obtained from these tumors.

Our investigations are now directed toward (*a*) the further elucidation of the nature of the active material, (*b*) the duplication of the effect of the growing tumor in the living embryo with the active material isolated from the tumors, and (*c*) an examination of the metabolic response of the nerve cells under the influence of the growth-promoting agent.

* This work has been supported in part by a grant from the National Institutes of Health of the United States Public Health Service. A preliminary report was presented at the forty-fifth annual meeting of the American Association for Cancer Research at Atlantic City, April, 1954.

[1] E. D. Bueker, *Anat. Record*, **102**, 369–390, 1948; R. Levi-Montalcini and V. Hamburger, *J. Exptl. Zool.*, **116**, 321–362, 1951; R. Levi-Montalcini, *Ann. N.Y. Acad. Sci.*, **55**, 330–343, 1952.

[2] R. Levi-Montalcini and V. Hamburger, *J. Exptl. Zool.*, **123**, 233–288, 1953.

[3] R. Levi-Montalcini, H. Meyer, and V. Hamburger, *Cancer Research*, **14**, 49–57, 1954.

[4] W. C. Schneider, *J. Biol. Chem.*, **176**, 259–266, 1948.

[5] S. S. Cohen, *J. Biol. Chem.*, **168**, 511–526, 1947; H. Von Euler and L. Heller, *Arkiv Kemi*, **26A**, No. 10, 1–16, 1948; R. J. Kutsky, *Proc. Soc. Exptl. Biol. Med.*, **83**, 390–395, 1948.

[6] O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265–275. 1951.

[7] W. C. Schneider, *J. Biol. Chem.*, **161**, 293–303, 1945.

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*ON TEMPERATURE INDEPENDENCE IN THE CLOCK SYSTEM CONTROLLING EMERGENCE TIME IN DROSOPHILA** †

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Clocks, processes measuring absolute time, occur in living organisms. Recently they have attracted interest, especially following the demonstration by Kramer[1] and Pardi and Papi[2] of the celestial navigation performed by birds and amphipods. The present paper is concerned with the clock system controlling the time at which *Drosophila* adults emerge from puparia. The timing of this event has attracted several earlier workers, especially Kalmus[3, 4, 5] and Bünning.[6] Kalmus' account of the temperature relations of the *Drosophila* clock, which is not confirmed here, formed an essential foundation for the present study and led to its being undertaken.

The temperature relations of organic clocks are of obvious interest. If a clock is to provide information involved in controlling important functions, then clearly it must be reasonably reliable. Attention is then focused on how reliable timing can be effected by poikilotherms, like gammarids and flies, in natural environments that are characterized by violent daily temperature variations. A temperature-dependent clock will guarantee only mistiming. Unlikely as it may seem on physical grounds, the biological prerequisite of temperature independence has been achieved by poikilotherms. This was shown more than twenty years ago, in a classical study of the bee's "time-sense," by Wahl.[7] Much more recently, Brown[8] has demonstrated temperature independence in the clocks controlling daily and tidal periodicities in *Uca* and *Venus*.