It contained 38,500 LBF units per ml and had a potency of 840 units per mg of dry solids. Diet 7 contained 3,200 LBF units in a daily supplement and was equivalent to 96 μg of bound calcium pantothenate as determined by alkaline hydrolysis and microbiological assay(7) of the β-alanine liberated. Paper chromatography of materials containing LBF has shown that 5 or more substances possess LBF activity(5,8). The concentrate used in this study consists mainly of a mixture of the 2 LBF's (Rf 0.83 and 0.74) obtained in concentrates from *B. megatherium* culture filtrates(8). Less than 2 μg of free pantothenate were contained in a daily supplement. Diet 8 contained twice these amounts of free pantothenate and pantothenate bound in the LBF molecule.

Results and discussion. There was no significant difference between the growth responses produced by the 4 highest levels of calcium pantothenate and either level of LBF concentrate at the end of 12 days. This merging of response is to be expected since a daily dose of 25 to 50 μg of Ca pantothenate appears to be adequate for optimum growth after the rat has recovered from severe depletion(6).

Diet 8 had a pronounced odor and was not readily consumed by the rats. Subnormal gains on this diet were probably the result of inadequate food intake due to unpalatability, although the possibility of toxicity due to impurities in the concentrates must also be considered.

Controls and some of the rats on the low pantothenate diets died in 3 to 4 days after the start of the experiment, as shown in Table I. All rats receiving over 50 μg of calcium pantothenate or the LBF supplements survived. This leaves little doubt as to the replacement of pantothenate by LBF for the rat. Further proof of this interrelationship is evidenced by the fact that rats which were prostrate after a depletion of 24 to 25 days were on their feet and had apparently recovered in a few hours after intraperitoneal injection with LBF concentrates or calcium pantothenate. Death occurred in 1 to 2 hours after prostration in uninjected rats.

Summary. Evidence is presented to show that concentrates of the *Lactobacillus bulgaricus* factor will replace pantothenic acid in the nutrition of the rat. The quantitative effect of LBF in promoting growth may be in excess of that expected from the pantothenate contained in the LBF molecule.

We are indebted to Dr. J. C. Lewis, Western Regional Research Laboratory, Albany, Calif., for the *B. megatherium* growth liquors and to Mr. C. L. Long of this department for the paper chromatographic analysis of the LBF concentrate.

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Occurrence of Tyrosinase in Horse and Fish Melanomas. (18210)

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In addition to the presence of melanomas in man, these tumors have been reported in the horse, mule, cow, swine, goat, dog, cat, chicken, rabbit, mouse, snake, and fish(1-6). De-

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spite this widespread occurrence in animals, particularly in horses(1,2), there are relatively few reports on the biochemical properties of these tumors. It has been found that extracts from horse melanomas(7,8) catalyze the conversion of tyrosine to melanin, and that extracts from the Harding-Passey mouse melanoma(9,10) catalyze the formation of melanin from both tyrosine and 3,4-dihydroxyphenyl-L-alanine (dopa). Alsberg(11) prepared an extract from a human melanoma which catalyzed the formation of black pigment from catechol and possibly from tyrosine. Neuberg(12) showed that dilute extracts from a human melanoma accelerated pigment formation from tyramine and epinephrine but not from tyrosine. Greenstein and others(13) found that extracts from a human melanoma showed both tyrosinase and dopa-oxidase activity. We have shown(14) that extracts of the Harding-Passey mouse melanoma are equally active in catalyzing the enzymatic oxidation of both tyrosine and dopa under the proper conditions, and we suggested that the general term "tyrosinase" be used for the enzyme or enzyme complex concerned instead of the more restrictive terms "tyrosinase" and "dopa-oxidase." We further demonstrated(15) that tyrosinase from mouse melanoma, like tyrosinase from plants(16) and insects(17), requires copper for its activity and is inhibited by diethyldithiocarbamate and other compounds having an affinity for copper.

This report describes the enzymatic oxidation of tyrosine and dopa by extracts of horse and fish melanomas, and the inhibition of this oxidation by diethyldithiocarbamate.

Experimental methods. Preparation of the tumor extracts. (A) Horse melanoma. The specimen,§ the largest of a group of firm nodular and ovoid tumors located in the skin of the ventral surface of the tail, anal and perineal regions, was removed from the right rectal fossa. The tumor weighed approximately 500 g, and was coal-black in color. After removal, the tumor was frozen with solid carbon dioxide until used 3 hours later. To prepare the extract, approximately 2 g of the melanomatous tissue were ground with sand and cold 0.1 M potassium phosphate buffer, pH 6.8, for 10 minutes. The resulting black, syrupy material was allowed to stand for 12 hours at 5°C. The watery supernatant fraction was then decanted from the large clumps at the bottom of the vessel and used for the enzymatic studies.

(B) Fish melanoma. The melanoma-bearing fish¶ used in the experiments were first generation hybrids between a male spotted-belly platyfish *(Platypoecilus maculatus)* and an albino swordtail *(Xiphophorus helleri)*. Gordon(6) has demonstrated that there is a very high incidence of spontaneous melanomas in such offspring.

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§ The gray mare from which this tumor was removed was supplied by Sharp & Dohme, Inc., Philadelphia, Pa., through the courtesy of Drs. R. H. Barnes and B. J. McGroarty. The animal was estimated to be 17 years old.

¶ The fish were obtained from the aquarium of the New York Zoological Society through the courtesy of Dr. Myron Gordon.

The fish were killed by chilling in ice and were kept in ice until used, 2 days later. The deeply pigmented tumors, each weighing approximately 50 mg, were removed by blunt dissection and were ground in sand and 5 ml of cold 0.1 M potassium phosphate buffer at pH 6.8 for 5 minutes at room temperature. The material was then centrifuged at 2,500 revolutions per minute for 20 minutes at 5°C. The clear, red-brown supernatant fraction, containing 0.5 mg of nitrogen per ml, was stored at 5°C until used one to 2 hours later.

Enzymatic oxidation of L-tyrosine and L-dopa was measured manometrically as oxygen uptake in the Warburg apparatus, at 38°C and in 0.1 M potassium phosphate buffer at pH 6.8, using 0.2 ml of horse melanoma extract or 0.5 ml of fish melanoma extract per vessel, in a total fluid volume of 3 ml. The substrates (tyrosine, dopa) were added in 0.5 ml of buffer from the vessel side arms after 10 minutes equilibration, and manometric readings made at 10 minute intervals thereafter. Sodium diethyldithiocarbamate was dissolved in buffer to give 0.3-0.5 mg per vessel and added directly to the enzyme solution before equilibration. In the experiments involving heavy metals in the presence of diethyldithiocarbamate, a 25% excess of the various metal salts, calculated on a mole for mole basis, was dissolved in 0.1 ml of water and added to the reaction mixture from a second side arm after the reaction had been allowed to run for 30 minutes. The metal salts used were cupric sulfate, ferric nitrate, magnesium chloride, zinc sulfate, nickelous sulfate, cobaltous sulfate and manganous chloride.

Results. Fig. 1 and 2 illustrate the varying rates of oxygen uptake when tyrosine, dopa, and tyrosine plus a trace amount of dopa, were used as substrates with extracts of the horse and fish melanomas. It is evident that the 2 extracts have qualitatively the same enzymatic activity. With dopa as the substrate, oxygen uptake began almost immediately. With tyrosine, however, oxygen uptake did not commence until after an interval of approximately 30 minutes (horse melanoma extract) and 90 minutes (fish melanoma extract). Substitution of 0.05 mg of dopa for an equal quantity of tyrosine markedly shortened this interval and there was a rapid oxidation of tyrosine. The added dopa by itself can account for an oxygen uptake of not more than 11 microliters as indicated by the dotted horizontal lines in Fig. 1 and 2.

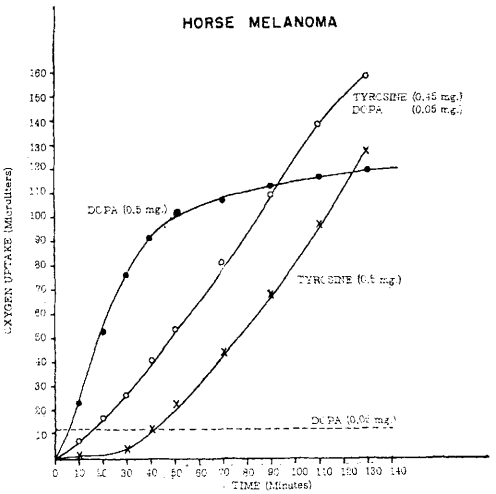

FIG. 1.

Enzymatic oxidation of tyrosine and dopa by an extract from the horse melanoma at pH 6.8 and 38°C.

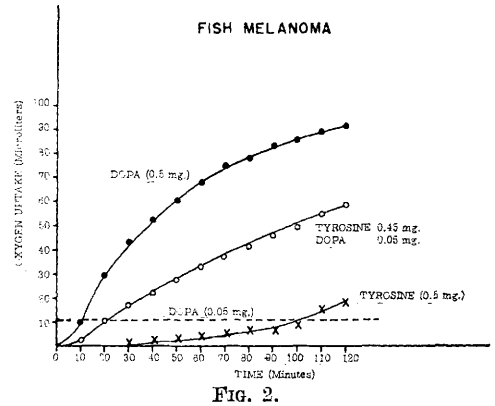

FIG. 2.

Enzymatic oxidation of tyrosine and dopa by an extract of fish melanoma at pH 6.8 and 38°C.

A preparation of the horse melanoma extract, made by the method described above, except that the extract was centrifuged at 3,000 r.p.m. for 30 minutes at 5°C, yielded a brown supernatant fraction which had no enzymatic activity. This suggests that en-

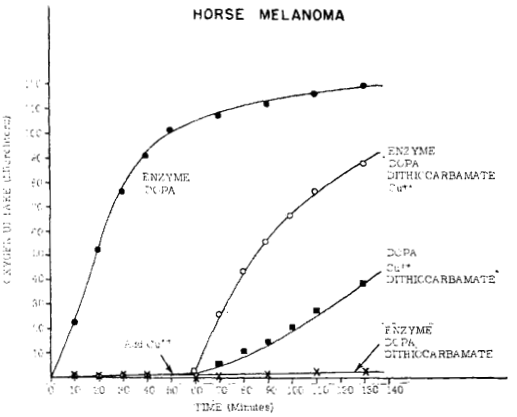

FIG. 3.

The effect of sodium diethyldithiocarbamate (0.5 mg) on the enzymatic oxidation of dopa (0.5 mg) by an extract from the horse melanoma and the reversal of inhibition by Cu++ (0.9 mg $CuSO_4 \cdot 5\ H_2O$); pH 6.8 and 38°C.

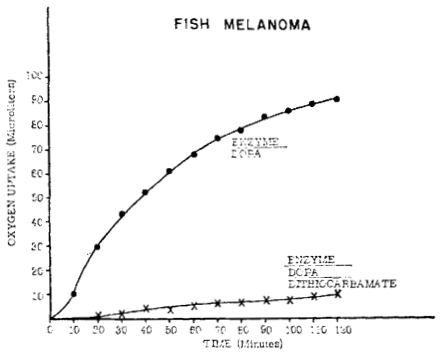

FIG. 4.

The effect of sodium diethyldithiocarbamate (0.3 mg) on the enzymatic oxidation of dopa (0.5 mg) by a fish melanoma extract at pH 6.8 and 38°C.

zymatic activity is associated with insoluble particulate matter of the cell, as has been shown for mouse melanoma(14). However, the mouse melanoma enzyme is not separated from the supernatant fluid under these particular conditions, indicating that the particulate material in the horse extracts is larger than that in the mouse extracts. Tissue slices of the horse tumor showed the same enzymatic activity as the uncentrifuged extract.

Sodium diethyldithiocarbamate almost completely inhibited the uptake of oxygen by mixtures of horse tumor extract and substrate. This was reversed by the addition of cupric ions (Fig. 3). The catalytic effect of cupric ions on the auto-oxidation of dopa is also illustrated. Reversal of diethyldithiocarbamate inhibition did not occur on the addition of manganous, ferric, magnesium, zinc, nickelous and cobaltous ions.

The marked inhibition of oxygen uptake produced by sodium diethyldithiocarbamate in the mixtures of fish tumor extract and substrate is shown in Fig. 4. An attempt to reverse this inhibition by the addition of an excess of cupric ions did not yield conclusive results. Unfortunately there was insufficient material for the detailed controls necessary to clarify this point. Attempts to demonstrate tyrosinase activity in extracts from normal pigmented and non-pigmented skin of fish of the same species yielded negative results.

The foregoing experiments demonstrate the presence of tyrosinase in extracts of horse and fish melanomas. The pattern of enzymatic activity is similar to that reported(14) for the tyrosinase in extracts of mouse melanoma, particularly with respect to the catalytic effect of dopa on the oxidation of tyrosine. Inhibition of tyrosinase activity by diethyldithiocarbamate, and its specific reversal by added cupric ions, strengthens the view that horse melanoma tyrosinase (and probably the fish melanoma enzyme also) requires copper for enzymatic activity, as does the mouse melanoma enzyme(15).

Summary. 1. A crude extract with marked tyrosinase activity has been prepared from melanomas of the horse and certain species of hybrid fish. These extracts catalyzed the oxidation of both tyrosine and dihydroxyphenyl-L-alanine (dopa) to melanin. In addition, dopa has a catalytic role in the enzymatic oxidation of tyrosine by these extracts.

2. Diethyldithiocarbamate, a substance with a strong affinity for copper, produces a marked inhibition of the enzymatic reaction. This inhibition is reversed by cupric ions in the case of the horse melanoma extracts. This reversal is not obtained with other metals.

3. The enzymatic activity of horse melanoma extracts appears to be associated with cellular particulate matter, which is larger than that found in mouse melanoma extracts.

4. The biochemical properties of the tyrosinase preparations from the horse and fish melanomas are similar to the properties of the preparation obtained from mouse melanoma.

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Effect of Adrenocorticotrophic Hormone, Cortisone and Desoxycorticosterone on Brain Excitability.* (18211)

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It has been demonstrated in this laboratory that desoxycorticosterone acetate (DCA) decreases brain excitability as measured by the electroshock seizure threshold (EST)(1,2), and that brain excitability of DCA-treated rats can be restored to normal by the administration of adrenocorticotrophic hormone (ACTH) or adrenocortical extract (ACE)(2). The following experiments are an extension and elaboration of these earlier studies.

Methods. Adult male rats from the Sprague-Dawley farm were employed. The technic for determining EST has been presented in detail(3). EST measurements were made at 2- to 3-day intervals for a period of about two weeks before the beginning of hormone administration. Thresholds tend to be more variable and slightly higher at the beginning than at the end of such a period of stabilization. Hence, the alterations in EST observed throughout the course of the experiments have been expressed as a percentage of the control value obtained at the *end* of the stabilization period. When thresholds had stabilized, some of the rats were unilaterally nephrectomized, implanted subcutaneously with DCA† (twelve 15-mg pellets per rat) and given a 0.9% solution of sodium chloride to drink. EST measurements were made every 2 to 3 days throughout the experiments. The dose of ACTH‡ is expressed in all instances as the mg equivalent of the ACTH standard, La-1-A. Preparation 74 Ax, employed in Experiment A, is 50% as potent as La-1-A; it has posterior pituitary pressor activity of less than 0.06 unit per mg equivalent of La-1-A. Preparation 83 ASI, employed in Experiment B, is 160% as potent as La-1-A; it has posterior pituitary pressor activity of less than 0.05 unit per mg equivalent of La-1-A. ACTH was dissolved in a 0.9% solution of sodium chloride at pH 1; each dose was injected subcutaneously in a volume of 0.2 ml. Cortisone acetate was suspended in 10% ethanol; each dose was injected subcutaneously in a volume of 0.2 ml. Posterior pituitary substance (Pitressin, Parke, Davis and Co., 20 pressor units per ml) was diluted with 0.9% sodium chloride solution to a concentration of 0.1 unit per 0.2 ml; this volume was injected subcutaneously. When combinations of hormones were employed, the preparations were injected throughout the entire course of the experiment, starting at the time of DCA implantation. This is in contrast to the previous study(2) in which ACTH and ACE were not injected until the rats had been under the influence of DCA for some time.

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‡ ACTH was obtained through the kindness of Dr. E. E. Hays of the Armour Co.