Synergistic Reaction Between *p*-Phenylenediamine and Melanoma Components.* (23939)

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An unaccountable oxygen uptake has been observed when melanoma extracts or homogenates are tested manometrically with *p*-phenylenediamine (PPDA)(1-4). This compound is ordinarily employed as a cofactor in Warburg measurement of cytochrome oxidase activity in tissue slices or extracts, and reduces cytochrome c to the enzymatically oxidizable or usable form. Melanoma homogenates, in contrast to most other tissues, are unique in oxidizing added PPDA by an unknown mechanism other than the cyanide sensitive cytochrome system. Specifically, when PPDA is added to non-melanoma tissue extracts deprived of cytochrome c, or otherwise inactivated, no significant oxygen consumption occurs beyond the slight autoxidation of PPDA itself, whereas with extracts from malignant pigmented melanoma a significant additional oxygen uptake is found (1-3). These observations are illustrated in Fig. 1 and are further discussed with experimental results. In experiments to elucidate this phenomenon it was discovered that an accepted melanoma metabolite, dihydroxyphenylalanine (DOPA), was capable of combining at physiological temperatures with PPDA in a relatively vigorous oxygen consuming reaction when the 2 purified compounds were combined *in vitro*. A preponderance of the resulting products were pigmented and grossly resembled natural melanin (Fig. 5). A small amount of CO_2 was also formed. The following experiments illustrate some characteristics of the reaction, evidence for its relationship to the above melanoma phenomenon, and some possibilities for its exploitation in diagnosis and chemotherapy of malignant melanoma.

Materials and methods. *p*-Phenylenediamine (PPDA), known also as Ursol and PPD, was received from several sources (Matheson, Coleman & Bell; Merck; Amend; and Eastman Kodak Co.). Although the autoxidative rates varied somewhat with source and purity (3), we noted no significant departures from the synergistic reaction reported here. Under some conditions certain differences are seen between the free base and the neutralized dihydrochloride; however, only the free base was employed in experiments of this report. 3,4 - Dihydroxyphenyl - DL-alanine (DOPA) was from Nutritional Biochemicals Corp. Its stock solution, as well as PPDA, was freshly prepared for each experiment. Oxygen consumption was measured in air by standard manometric procedures at 38°C with the vessels shaken at 112 cycles/minute and an amplitude of 4 cm. Reagent concentrations are expressed in molar terms and were calculated as final concentrations in the manometric vessel following tipping of sidearm component. Other pertinent details are given with the descriptions of specific experiments or are contained in the Figures.

Results. The experiment illustrated in Fig. 1 demonstrates several types of oxygen consumption when a cell-free Cloudman S91 mouse melanoma extract containing enzymically active melanin granules and the usual supernatant components(5) was tested manometrically under various conditions with and without PPDA. The upper 2 curves are persuasive in demonstrating that the reaction obtained when PPDA was added to melanoma extracts is probably not enzymatic in nature since the oxidation factor associated with the tumor is both KCN and heat insensitive. The inactivated fractions contained the same components as the nearly vertical curve depicting the cytochrome oxidase activity of the extract except for the

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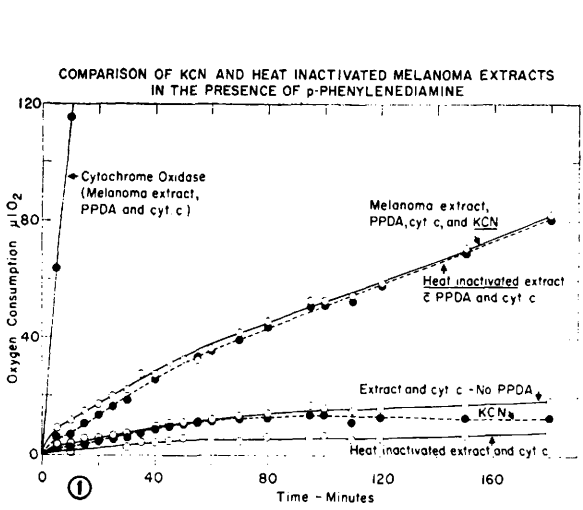

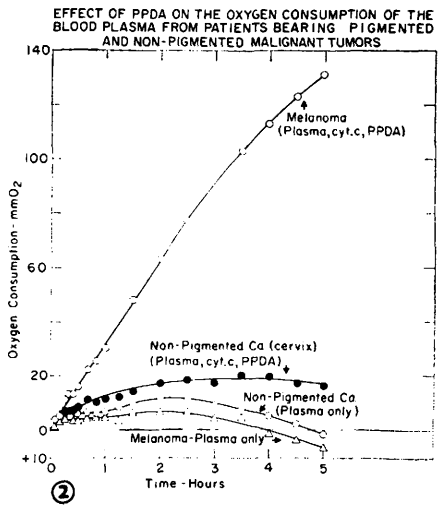

FIG. 1. Non-enzymic oxidation of *p*-phenylenediamine (PPDA) in KCN and heat inactivated mouse melanoma extract. (0.5 ml of 10% Cloudman S91 aqueous extract; phosphate buffer, 0.05 M, pH 7.4; cytochrome c, 5×10^{-5} M; PPDA, 0.05 M; final vessel volume, 2 ml.)

FIG. 2. Differential oxygen consumption of plasma from a melanoma patient compared with plasma from a patient with a non-melanotic malignancy. (Heparinized plasma; 0.02 M PPDA; 0.04 M phosphate buffer, pH 7.4; cytochrome c, 5×10^{-5} M but not essential.)

addition in one case of KCN to give a final vessel concentration of 0.003 M. The nearly identical broken line curve shows the similarity of oxygen uptake when the enzymes of the extract had been denatured by heating in boiling water for 10 minutes. The congruity of the two inactivation curves also indicates that essentially all oxidative enzyme activity in this melanoma extract is cyanide sensitive. The enzymatic dependence on an added cytochrome c reductant is shown by the lower endogenous oxidation curve where all necessary components for cytochrome oxidase activity are present except PPDA, or such equivalents as ascorbic acid or cytochrome c reductase. This particular oxidation-reduction state of melanoma tissue is probably related to the high differential PPDA response phenomenon described by Burk, *et al.* for this tumor(6). There is, of course, no significant oxygen consuming reaction between PPDA and cytochrome c at the concentrations usually employed in enzyme assays or in these experiments(3).

A reaction similar to that seen with melanoma extract has repeatedly been observed with the blood and urine of patients with advanced pigmented melanoma. Fig. 2 illustrates the differential oxygen consumption obtained when PPDA is added to the plasma of a patient with disseminated pigmented melanoma as compared with a patient in a comparable stage of a non-melanotic malignancy. Although the identity of this PPDA reacting substance has not been established by isolation it seems permissible to assume that it is a metabolic product of the extensive pigmented metastases present in the patient. This reaction provides the basis for a possible diagnostic procedure and is being further studied. One of the difficulties to be overcome is the occasional presence in non-melanoma urines and plasmas of interfering substances that are capable of giving false positives.

The time-course curves of the oxygen consuming reaction when PPDA was added to DOPA and closely related compounds at pH 7.4 are shown in Fig. 3. It may be seen that the two ortho hydroxyl groups are essential for the reaction since neither tyrosine nor phenylalanine yields a significant uptake with PPDA, whereas pyrocatechol does give a partial uptake amounting to approximately half that obtained when the alanine side chain is also present. Whether the alanine plays a role in feeding electrons to the reaction sites or merely assists in molecular orientation in the formation of the resulting complex is un-

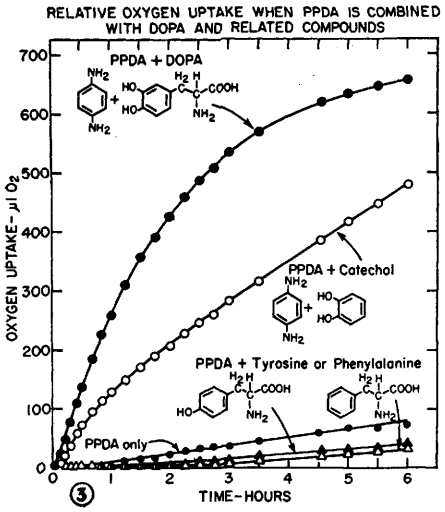

FIG. 3. Relative oxygen uptake when PPDA is combined with DOPA and related compounds. (PPDA, DOPA, catechol, phenylalanine, 0.01 M; 0.05 M phosphate buffer, pH 7.4.)

certain. Although no systematic study has been made of compounds related to DOPA, approximately 150 PPDA-related aromatic amines were examined for specific combination with DOPA from the standpoint of manometric oxygen consumption. Only a few were found active under the experimental conditions employed successfully with PPDA. The significantly active compounds were *p,p'*-diaminodiphenylamine, *p,p'*-diaminodiphenylamine-*o*-sulfonic acid, 2,4-toluenediamine, 2,3-diaminotoluene, 2,4-diaminobenzoic acid, and N,N-dimethyl-*p*-phenylenediamine.

Fig. 4 illustrates the activity curve obtained by varying the molecular ratio of DOPA to PPDA. While high activity is obtained at ratios of 1:1, 2:1, and 3:1, the peak of activity, at 1 hour, occurs at 2:1, which, together with other data, suggests the formation of a complex, possibly a polymer composed of the basic molecules of DOPA and PPDA. The detailed chemistry of this reaction is still under investigation. Fig. 5 shows

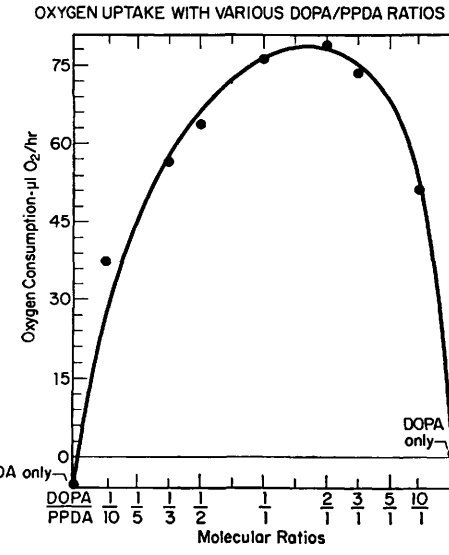

FIG. 4. Oxygen uptake as a function of DOPA-PPDA molecular ratio. (DOPA and PPDA, 0.01 M; phosphate buffer, 0.05 M, pH 7.4.) No NaOH employed for CO_2 absorption.

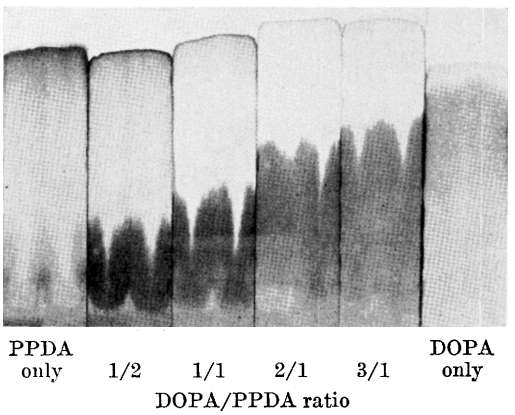

FIG. 5. Individual chromatograms of the pigment produced by various DOPA-PPDA ratios. (PPDA and DOPA, 0.01 M; phosphate buffer, 0.05 M, pH 7.0.)

the relative amounts of pigment formed in the above experiment and its differential behavior in a simple chromatographic system employing a type of capillary analysis(7,8). In contrast to the hyperbolic peaking of the oxygen consumption curve, the Rf values of the migrating pigment increase to the right with increasing DOPA as long as catalytic amounts of PPDA are present. Correlated with this, it was noted that the pigment formed with higher ratios of PPDA precipitated more readily, forming insoluble aggregates in the bottom of the chromatographic beaker.

The detection of an *in vitro* oxygen consuming reaction involving an intracellularly produced metabolite of melanoma tissue sug-

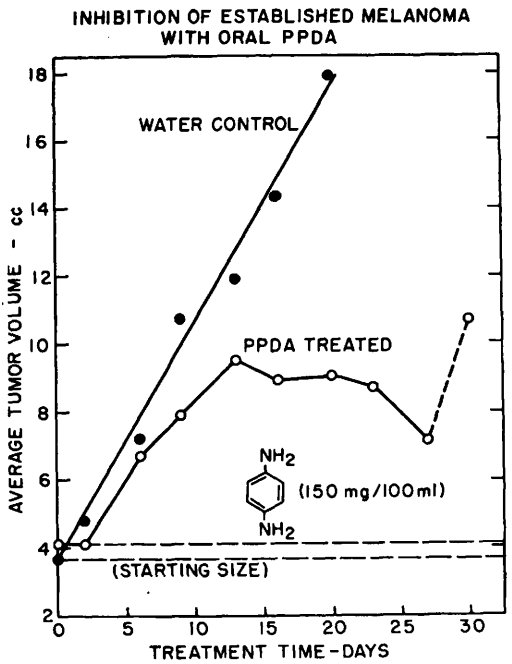

FIG. 6. Inhibition of established mouse melanoma with oral PPDA. (PPDA, 150 mg/100 ml of drinking water; tumor, 27-day post-implant of Cloudman S91 melanoma.)

gested the possibility of interfering selectively with the metabolism of this malignant cell if the reaction could be generated *in vivo*. Fig. 6 shows the inhibition of established Cloudman S91 mouse melanoma when hosts were treated orally with 150 mg of PPDA per 100 ml of drinking water. Treatment was started 27 days after implant of the tumors when the average tumor volume was approximately 4 cc. The tumor sizes illustrated are the means of 10 tumors in each group at the time treatment started, with the number decreasing to 1 or 2 animals at the end of the experiment. The treated animals survived approximately 30% longer than the controls. It should be noted that the ortho and meta isomers gave significantly greater melanoma inhibition. Further details on these compounds will be described separately.

Discussion. Although PPDA and its oxidation products have been implicated in certain combinations with enzymes and other proteins (9,10), data recently reported from this laboratory provide evidence of a special affinity of PPDA for melanoma tissue or its products (11). Specifically, when melanoma bearing mice are injected with an acutely lethal dose of PPDA they survive significantly longer than do their non-tumor controls. In view of the findings reported here the most consistent explanation of this phenomenon seems to be an *in vivo* detoxification of the injected PPDA by its combination with DOPA-like components present in the melanoma tissue (Fig. 1) or in the peripheral blood (Fig. 2), or by both, since the products of similar combinations incubated *in vitro* are less toxic to the host than are the individual components. The chemotherapy experiments with transplanted pigmented tumors were stimulated by the above data and its implications. However, the verification of a mechanism whereby an intratumoral combination of PPDA with DOPA-like metabolites disrupts the metabolic integrity of the malignant cell and thus causes its inhibition or destruction is a complicated undertaking and necessitates further study. At present the most persuasive argument in its favor is the observation of tumor inhibition by PPDA and related compounds.

Summary. A relatively vigorous oxygen consuming, non-enzymic reaction occurs at physiological temperatures between *p*-phenylenediamine (PPDA) and dihydroxyphenylalanine (DOPA) when the 2 compounds are mixed in aqueous solution at pH 7.0 to 7.5, with maximum activity, based on oxygen consumption, obtained at a DOPA-PPDA molecular ratio of approximately 2:1. The reaction has been studied manometrically with pure compounds and with various melanoma components, such as are contained in mouse melanoma extract and in plasma and urine from patients with disseminated malignant melanoma. The oxidation observed with various melanoma components is thought to be identical or analogous to the PPDA-DOPA reaction found with the pure substances since in all instances the reaction is both KCN and heat insensitive. Preliminary data are given to illustrate the possibilities of exploiting such reactions for diagnostic and chemotherapeutic purposes.

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Production by Various Fats of Myocardial Necroses in Humorally Conditioned Rats.* (23940)

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When rats are simultaneously treated with certain highly active corticoids, such as 2α-methyl-9α-chlorocortisol (Me-Cl-COL)† plus sodium phosphates (Na_2HPO_4, NaH_2PO_4), they invariably succumb with clinical manifestations of acute cardiac death, and autopsy regularly reveals large, yellowish infarct-like necrotic patches in their myocardium(1). Exposure to stress (*e.g.,* neuromuscular exertion) can precipitate the development of such infarct-like myocardial necroses, in rats conditioned by pretreatment with small doses of corticoids and electrolytes(2). These cardiac lesions are not accompanied by any morphologically demonstrable evidence of coronary artery occlusion, yet they resemble cardiac infarcts in some respects. Since, during the last few years, a great deal of evidence has accumulated in support of the concept that certain fats may precipitate the development of true cardiac infarcts in man(3), we wanted to verify whether the production of myocardial necroses by corticoids plus sodium phosphate in the rat could likewise be precipitated by the oral administration of fats.

Materials and methods. One hundred forty female Sprague-Dawley rats, with an average initial body-weight of 99 g (range: 93-109 g), were subdivided into 14 equal groups as indicated in Table I. Me-Cl-COL was injected subcutaneously, in the form of its acetate, as a micro-crystal suspension of 100 μg in 0.2 ml of water, once daily. Na_2HPO_4 was administered by stomach tube, at the dose of 1 mM (141.98 mg) in 5 ml of water, twice daily. The various fats were given twice daily, by stomach tube, at the doses indicated in Table I. The experiment was terminated after 6 days, by killing the survivors with chloroform. The hearts of all animals were fixed in Susa solution, for subsequent staining with hematoxylin-eosin. Since nephrocalcinosis frequently accompanies the myocardial necroses produced by Me-Cl-COL plus Na_2HPO_4, specimens of all kidneys were fixed in neutral formalin for the subsequent histochemical demonstration of calcium deposits by the v. Kossa's silver nitrate technic. Both organs were embedded in paraffin.

Results. The principal results of these experiments are summarized in Table I, which lists the mean final body-weight, the severity (graded in terms of an arbitrary scale of 0 to 3) of cardiac necroses and nephrocalcinosis, as well as the percentual incidence of these changes, and the mortality rates.

Treatment with as much as 1 ml of corn oil, twice daily, alone (Group 1) or in com-

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