gland adsorbs ACTH when present in the blood in increased concentration. Gemzell(5) has claimed that administration of ACTH for one week to adrenalectomized rats was followed by no change in pituitary ACTH content. His results are not comparable to those herein described since his technics(3) of pituitary ACTH extraction and assay yielded values 80% lower than those obtained in the present report.

Summary. A modification of the Saffran and Schally technic of *in vitro* bioassay of ACTH is described and evidence is presented for its precision, validity and reliability. Changes in pituitary ACTH content and concentration in rats after administration of various hormones for one week were studied by means of this technic. ACTH administration and adrenalectomy both resulted in a striking increase in pituitary ACTH content. Epinephrine and cortisone administration were both followed by significant depletion of pituitary ACTH.

ACTH was supplied through the kindness of Dr. K. W. Thompson, of Organon, Orange, N. J. The authors wish to express gratitude to Dr. C. I. Bliss for invaluable advice concerning statistical analyses. Miss Myra Feder provided expert technical assistance.

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Demonstration of Differences Between Normal and Tumor-Bearing Animals.* (23678)

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If it is assumed that tumors are metabolically or otherwise different from the normal host tissue, this distinction should express itself qualitatively or quantitatively in some of the intermediate or terminal biochemical products of malignant growth. A simple experimental design has been employed to demonstrate such differences in animals bearing transplanted tumors of various types in comparison with otherwise identical normal control animals. The principle of the procedure involves the challenging of tumor-bearing and normal mice with an intraperitoneal lethal dose of an appropriate compound capable of reacting *in vivo* with one or more of the unique metabolites associated with the tumor in question. The effect of this reaction on the survival times of the 2 groups of animals is then determined. Dissimilarity in survival times is presumably due to a decrease or in-

crease in the toxicity of the administered compound which, in turn, is thought to be an expression of its selective binding or chemical alteration by the tumor or its products. This measurable difference, as well as other variations in response, reflects the biochemical differences in the tumor-bearing hosts as contrasted with the controls.

The mouse melanoma has been employed as the primary model since some of its aberrant metabolic products and reactions are known. For example, 3,4-dihydroxyphenylalanine (DOPA) combines *in vitro* with *p*-phenylenediamine (PPDA) in an oxygen-consuming, pigment-producing reaction(1-4). DOPA is an accepted component produced in the biochemical chain of events in the metabolism of pigmented melanomas(5), and thus a rational foundation is provided for an *in vivo* reaction when PPDA is administered to melanoma-bearing animals. The consequence of this procedure, with the above combination, is a significant difference in the mean survival times of the two groups with a protective effect provided by the presence of the tumor. This phenomenon can be inverted by changing the administered compound or by employing a different histological or physiological type of tumor, in which event the tumor may become a biochemical liability to the host.

Materials and methods. The Cloudman S91 mouse melanoma(6), the Ehrlich (solid) carcinoma(7), and Sarcoma 180(8) were some of the tumors tested and are used illustratively for this brief report. The compounds reported include 3,4-dihydroxyphenyl-*DL*-alanine (DOPA), from Nutritional Biochemicals Corporation; *p*-phenylenediamine (PPDA), from Amend Drug Co. and Matheson, Coleman & Bell; N, N-dimethyl-*p*-phenylenediamine (DPP), from Eastman Organic Chemicals; and O-diazoacetyl-*L*-serine (Azaserine) and its analog, 6-diazo-5-oxo-*L*-norleucine (DON), from Parke, Davis & Co. The doses of the compounds are given in the text for the specific experiment but in general they were approximately 2 or 3 times the LD_{50}. All compounds were given as a single intraperitoneal dose on a milligram per kilo basis, including the weight of the tumor in the experimental group. Each mouse was weighed individually to 0.1 g for dose determination. The ratio in ml of the volume injected was 1/100 the weight of the animal. For convenience of dose calculation and injection the following standard procedure was employed. One hundred mg of compound was dissolved or suspended in 10 ml of water to give a dose of 100 mg/kg in a 20-g mouse when injected with 0.2 ml of solution. A 25-g mouse received 0.25 ml, etc. The dose volume was kept approximately constant for all compounds and the different requirements of milligrams per kilo to yield a lethal dose were controlled by an appropriate increase or decrease of drug concentration. All experiments were carefully balanced with regard to such tangible variables as time factors, and sex, strain, weight, and age of animals. To rule out or to detect the influence of time or of oxidation on the test solution, the experimental and control animals were injected alternately. When 2 or more tumor types or 2 or more compounds were being compared, appropriate alternation sequences were employed. The animals were identified by sequential numbering at the time of injection. Injection and death times were recorded to the nearest minute. Following death, the animals were reweighed and the tumors carefully removed to provide information on comparative tumor and carcass weights. For simplified analysis of the results the survival times were calculated to the nearest minute, the

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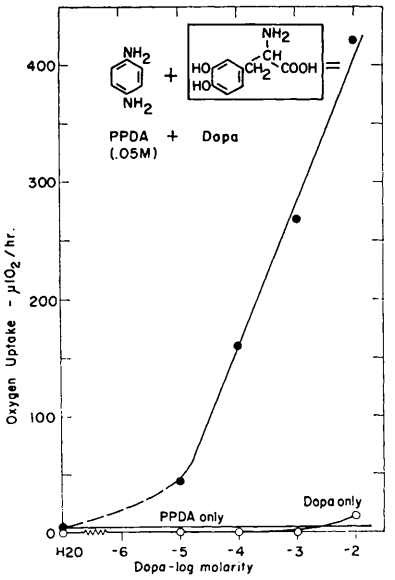

FIG. 1. Synergistic oxidation between 3,4-dihydroxyphenylalanine (DOPA) and *p*-phenylenediamine (PPDA) *in vitro*. PPDA concentration was constant at 0.05 *M* while DOPA was increased logarithmically.

data of the normal controls and the tumor-bearing mice were segregated, and the individual values within each group were arrayed in order of survival rank. For purposes of plotting the data and visualizing the family response of each group, cumulative averages have been employed. This procedure reduces the influence of one or 2 aberrant animals whose survival response is atypical. Thus, the first point for each group on the graph represents the shortest survival time of a single animal, while the next point represents the average survival time of the first 2 mice to die, etc. The last point represents the average survival time of all mice in its respective group. The midpoint on the curve, or the median average of the group response, is a more stable single value than the total average since, again, when the survival times are ranked, the median is independent of a small number of atypical longtime survivors. The experiments illustrated have in most cases been repeated several times with similar findings. Where statistical significance is of consequence it is discussed with the experimental results.

Results. Fig. 1 illustrates the manometric oxygen-consuming reaction that takes place when DOPA (3,4-dihydroxyphenyl-DL-alanine) and PPDA (*p*-phenylenediamine) are combined *in vitro*. It is seen that, under the conditions of the experiment, neither component oxidizes appreciably by itself but upon combination there is a vigorous oxygen uptake comparable to that produced by a potent enzyme preparation. In the illustrated experiment PPDA was held constant at 0.05 *M* while DOPA concentration was increased logarithmically to yield a straight line function through its solubility range. It is of consequence to note that a substantial uptake is still obtained when DOPA is present at the relatively low concentration of 1×10^{-4} *M*. A brown melanin-like pigment is the main product of the reaction and is less toxic to mice than the original components.

The protective effect of the S91 Cloudman mouse melanoma against the toxicity of PPDA is demonstrated in Fig. 2. The difference in the response between the two groups is highly significant and could occur by chance in less than 1 out of 100 cases ($P = 0.006$). The experiment was carried out with a uniform group of DBA/2 backcross female mice approximately 3 months old, with the only tangible difference between the 2 groups being the presence of melanoma which had been implanted 40 days previously. Both groups appeared to be in good physical condition. The normal controls had an average weight of 20.6 g and the tumor-bearing mice an average of 21.8 g. The tumors ranged in weight from 4.0 to 7.6 g with an average of 5.5 g. At death, when the tumors were removed, the carcass weights averaged 16.3 g.

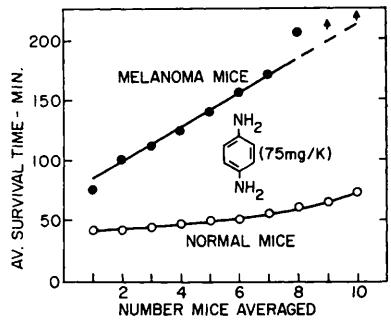

FIG. 2. Protective effect of Cloudman S91 melanoma against toxicity of *p*-phenylenediamine (PPDA).

TABLE I. Comparative Survival Time Response of Normal and Melanoma-Bearing Mice when Challenged with a Lethal Dose of PPDA (75 mg/kg).

	Normal control mice	Tumor-bearing mice	T/N ratio*
No. of mice	10	10	
Survival time			
Avg	72	289	4.0
Range	42–144	76–>640	
Stand. dev.†	30	195	
Median	60	222	3.7
Avg median‡	50	148	3.0
Avg mouse wt	20.6	21.8	
" carcass "	20.6	16.3	
" tumor "		5.5	

* Survival time ratio between the normal control and tumor-bearing mice.

† $P = 0.006$.

‡ Median point on the curves employing cumulative averages such as Fig. 2. This is the mean response of the first 50% of the animals in each group.

The experiment illustrated in Fig. 3 shows the dependence of average survival time upon tumor size. This experiment is similar to the preceding one but compared groups of small, medium, and large tumors with normal controls. The average tumor weights were 0.74, 1.81, and 5.6 g, with 10 mice in each tumor category and a total of 30 control animals. The differences are highly significant statistically and demonstrate the existence of a quantitative relationship between PPDA toxicity and the protective effect of melanoma in mice.

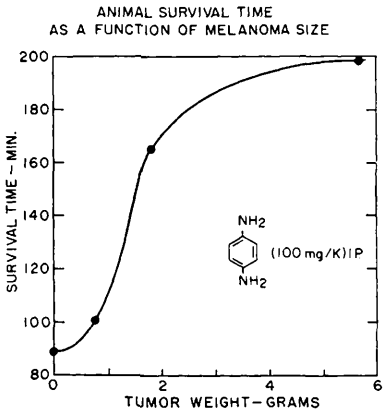

FIG. 3. Increasing protective influence of S91 melanoma as a function of tumor size against relative toxicity of an LD_{100} dose of *p*-phenylenediamine (PPDA).

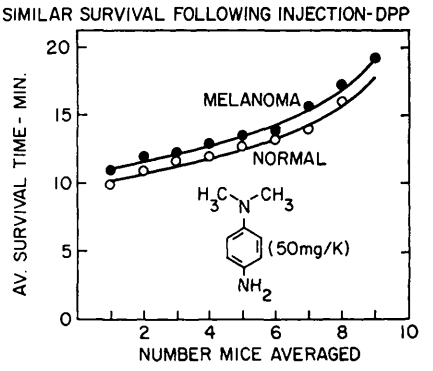

FIG. 4. Non-protective effect of S91 against toxicity of N,N-dimethyl-*p*-phenylenediamine (DPP).

If the hydrogens of one of the amino groups of PPDA are replaced by methyl groups to give N, N - dimethyl - *p* - phenylenediamine (DPP), the protective effect of the pigmented tumor against the compound's toxicity is lost (Fig. 4). Similar results were obtained with the nonpigmented S91 amelanoma. In a preliminary experiment with the same compound, animals bearing the Ehrlich carcinoma succumbed much sooner than did their normal counterparts to a lethal dose of DPP. When the Ehrlich tumor was tested with

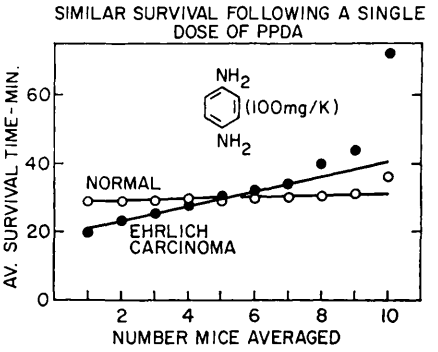

FIG. 5. Non-protective effect of Ehrlich carcinoma against toxicity of *p*-phenylenediamine (PPDA).

PPDA, however, in contrast to the differential toxicity obtained with melanoma, neither tumor-protective nor liability effects were observed (Fig. 5). It is thus seen that the various effects are dependent upon tumor type as well as upon compound structure.

Diaminodiphenylamine (DADPA) reacts with DOPA in the Warburg apparatus similarly to PPDA in that oxygen is consumed and pigment is formed. However, when

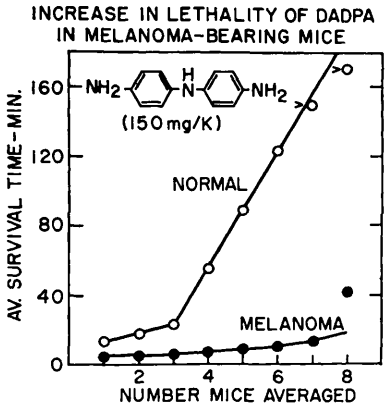

FIG. 6. Increased toxicity of diaminodiphenylamine (DADPA) in S91 tumor-bearing mice compared with normal controls.

tested against melanoma *in vivo* (Fig. 6), the opposite toxicity effect is obtained. Thus, with this compound, the tumor represents a liability rather than a protection to the organism, which indicates that the product resulting from DADPA and melanoma components is more–rather than less–toxic to the organism than the analogous products resulting from PPDA.

A striking difference in susceptibility to the acute toxic effects of Azaserine between normal Swiss mice and those carrying Sarcoma 180 is shown in Fig. 7. The average median survival time of the normal control animals was 598 minutes compared with 205 minutes for the tumor-bearing mice. Here again the presence of the tumor increases the toxic effect of the challenging compound. This is in sharp contrast to the finding with an analog of Azaserine, 6-diazo-5-oxo-*L*-norleucine (DON). The molecular difference between these two compounds resides in the substitution of a CH_2 for an oxygen atom, which change is sufficient to invert the influence of the tumor on the relative toxicity of the two compounds. Fig. 8 indicates the magnitude of the difference in response between control and experimental mice when injected with 400 mg/kg of DON and shows the protective effect provided by transplanted Sarcoma 180 tumors having an average weight of 1 g.

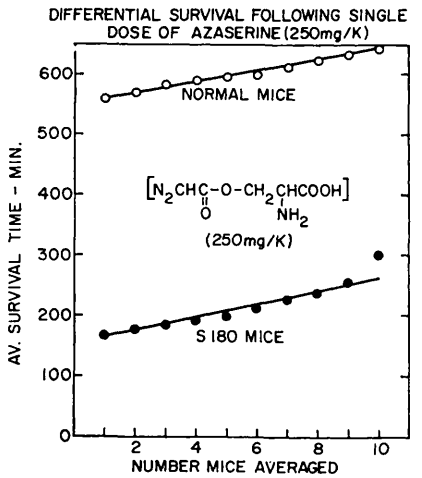

FIG. 7. Increased toxicity of Azaserine in Sarcoma 180-bearing mice compared with normal controls.

Discussion. The exact mechanism of the protection, or liability, which the tumor provides the host in the various circumstances cited above is uncertain. Some of the possibilities are as follows: 1. The toxic compound targets on the tumor and is physically adsorbed, chemically bound, altered, or otherwise detoxified or enhanced at the tumor site. 2. The injected compound is detoxified or its toxicity enhanced in the peripheral blood stream by specific tumor metabolic products present only in the cancerous animal. 3. There is a differential presence or absence of specific enzymes in the tumor-bearing animal, as compared with the normal controls, capable of altering or degrading the injected compound to a more or to a less toxic form.

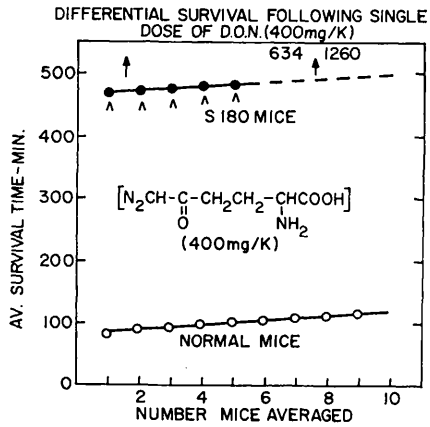

FIG. 8. Protective effect of Sarcoma 180 against toxicity of 6-diazo-5-oxo-*L*-norleucine (DON).

In experimental data in Table I it is seen that the average total mouse weight is approximately equal in the experimental and

control animals (20.6 and 21.8 g). When the tumors were removed, however, the average carcass weight of the experimental group was reduced to 16.3 g. Since the drug dose was based on total weight, the compound concentration would be expected to be equal in the carcasses of the 2 groups if the relative uptake by tumor and carcass were the same. A selective uptake or rejection of the drug by the tumor, however, would modify its concentration in the more vital carcass portion of the animal and thus, in turn, influence the survival span of the organism. The above experiments do not establish the site at which the injected compound is acted upon, but the data of Fig. 2 and 3 demonstrate that the tumor-bearing animals survived longer—as though the tumor, or its products in the peripheral blood, were selectively adsorbing or detoxifying the injected compound and thus protecting the tumor-bearing carcass at the expense of the tumor.

A similar interpretation can be made for the Sarcoma 180 experiments illustrated in Fig. 7 and 8 where diametrically opposite results were obtained with Azaserine and DON. This is an example, however, where the intervention of a tumor enzyme could be involved since it has been reported that Sarcoma 180 has an enzyme that can destroy Azaserine but not DON(9). If such an enzymic destruction of Azaserine resulted in a more toxic product for the host, it could account for the striking increase in vulnerability of the tumor-bearing animals when challenged with this compound as compared with DON. Other possibilities must also be considered, such as liver dysfunction, which may occur in Sarcoma 180-bearing mice. Since the liver sometimes becomes a detoxifying site for certain substances, a differential survival such as that observed with Azaserine (Fig. 7) could be due to an inability of the liver to detoxify the compound. In such instances the liability effect of the tumor might be an indirect one. Differential uptake between DON and Azaserine in Sarcoma 180 does not seem to be the explanation since uptake studies with ascites tumor cells showed no difference, though the tumor and the liver concentrated these compounds substantially more than did skeletal muscle(10).

The findings reported here, together with other unpublished data, indicate that the notion that tumor-bearing animals are more vulnerable to chemical challenge because they are in a "weakened" condition is an incomplete concept and may lead to errors in the determination of maximum tolerable doses. The experiments illustrated in Fig. 7 and 8, for example, suggest that the maximum acute tolerable dose of Azaserine determined in normal, non-tumor-bearing mice would be too high for mice bearing Sarcoma 180 and, on the contrary, would be too low if the determination were for DON. In the case of PPDA and the S91 melanoma the presence of a medium-sized tumor neutralizes or detoxifies as much as one-fourth of the dose when it is administered at 100 mg/kg. That is, in a comparative experiment, the tumor-bearing animals survived as long as the normal controls which received only 75 mg/kg. Although these experiments were performed with high doses for analytical convenience, similar phenomena can be obtained at lower doses, in the vicinity of the LD_{50}, and the differences between tumor-bearing and normal animals can be measured on a percentage survival basis.

Most investigators would probably agree that cancer chemotherapy involves the successful exploitation of critical differences between normal and neoplastic tissues. The chronological phases in a systematic rational approach would appear to be detection, elucidation, and utilization of such differences. The procedures briefly reported here are capable in their present form of detecting certain subtle distinctions of a biochemical nature between various tumor-bearing animals and their normal counterparts. Upon extension, these procedures may also be helpful in elucidating those distinctions. Empirically, the mere finding of a differential response is an adequate basis for undertaking chemotherapeutic studies, whatever the explanation or mechanism of that response. However, for purposes of experimental design, a special assumption may be derived from these findings, namely, that certain specific tumor products are expressions of a metabolic distinction of

particular types of neoplastic cells and that such endogenous components could constitute a lethal accumulation for the malignant tissue if ignited by the proper reactant.

Summary. A simple experimental procedure is reported which can demonstrate a significant difference in response between certain tumor-bearing mice and their normal controls. The procedure involves challenging the two groups of animals with a lethal dose of a compound capable of reacting with the tumor or one of its metabolites. Either the cancerous or the normal animals may survive longer, depending upon both the type of tumor and the molecular configuration of the compound utilized. The difference in survival time between the tumor-bearing and normal animals is thought to be due to a modification of the toxicity of the administered compound following reaction with a tumor component. The S91 mouse melanoma was employed as the primary model since some of the chemistry of its metabolites is known. Implications of possible utility in the study of the biochemistry of tumors and in chemotherapy are discussed.

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Renal Clearance of Ribonuclease. (23679)

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Rabinovitch and Dohi have reported an increase in the serum concentration of ribonuclease (RNAase) in bilaterally nephrectomized dogs(1). Using a new simple assay procedure for determination of RNAase in serum and urine(2), we have measured its renal clearance in normal subjects, including 5 humans and 3 dogs. In addition, observations have been made on the arteriovenous concentrations of RNAase across the intact canine kidney, as well as the changes in serum RNAase concentration following bilateral ureteral ligation.

Materials and methods. Crystalline pancreatic RNAase was obtained from Armour and Co. The activity of the enzyme was assayed upon yeast ribonucleic acid (RNA) from the California Foundation for Biochemical Research. Serum levels of RNAase were determined by diluting 0.1 ml of serum to 10 ml with a solution containing 0.02M NA_2HPO_4 and 0.14M NaCl. 0.5 ml of this solution was added to a similar volume of saline-phosphate containing 1 mg of RNA per ml. The pH remained constant at 7.8 during subsequent incubation of this mixture at 37°C for 30 minutes. After this period, 5 ml of 0.1M acetate buffer, pH 3.9, containing 3 mg/ml of bovine serum albumin (Fraction V, Armour) and 1 mg/ml of Knox gelatin was added to each mixture. The absorbence of the turbid colloid resulting from the interaction of the polymerized RNA with the acidified serum albumin was determined in a Coleman Jr. spectrophotometer at 400 mμ. The optical density so determined was translated by a standard curve into μg/ml of polymerized RNA remaining. The difference between this value and the initial 500 μg was expressed as μg of substrate depolymerized.