The results presented in Table 1 also suggest that indole oxidation is a property of bacterial dioxygenases that form *cis*-dihydrodiols from other aromatic hydrocarbons. *Beijerinckia* strain B836 is a mutant that oxidizes biphenyl and various polycyclic aromatic hydrocarbons to *cis*-dihydrodiols, whereas *P. putida* strain 39/D oxidizes benzene, toluene, and several monocyclic aromatic hydrocarbons to *cis*-dihydrodiols (*4, 11*). Both of these mutant strains and their wild-type parents oxidize indole to indigo. The ability of both B836 and wild-type *Beijerinckia* strains to oxidize indole to indigo is induced by *m*-xylene. The enzyme system from *P. putida* that catalyzes the formation of (+)-*cis*-(1*S*,2*R*)-dihydroxy-3-methylcyclohexa-3,5-diene (*cis*-toluene dihydrodiol) from toluene has been resolved into three protein components that are essential for enzymatic activity (*11*). This enzyme system consists of a flavoprotein (ferredoxin$_{TOL}$ reductase), a two-iron–two-sulfur ferredoxin (ferredoxin$_{TOL}$), and an iron-sulfur protein (ISP$_{TOL}$). Mutations in any one of the structural genes that code for these enzymes results in a complete loss of toluene dioxygenase activity (*12*). These mutants—strains F106, F102, and F26A (Table 1)—are also incapable of oxidizing indole to indigo.

The reaction sequence shown in Fig. 2 accounts for the formation of indigo by the recombinant *E. coli* and the other bacterial strains used in this investigation. It also provides a possible explanation for previous reports on the bacterial formation of indigo (*13–15*). We have not been able to detect the formation of *cis*-2,3-dihydroxy-2,3-dihydroindole. However, the formation of this intermediate can be inferred from the results presented in Table 1. Elimination of water from the *cis*-dihydrodiol would yield indoxyl, which is a known precursor of indigo. In addition, we have detected the presence of oxindole in culture filtrates after indole transformation by *P. putida*. Oxindole, the keto tautomer of 2-hydroxyindole, is the other expected product after dehydration of *cis*-2,3-dihydroxy-2,3-dihydroindole. The sequence in Fig. 2 differs from the reactions proposed by Fujioka and Wada (*16*) for the oxidation of indole to 2,3-dihydroxyindole by a Gram-positive coccus. However, these authors reported the accumulation of an unidentified blue pigment by indole-grown cells.

Our results illustrate the potential of recombinant DNA technology in the development of innovative microbial methods for the production of useful chemicals. The cloning and expression of naphthalene dioxygenase genes in *E. coli* will facilitate studies on the regulation and expressions of genes involved in the microbial degradation of aromatic hydrocarbons. In addition, the observation that indigo formation is catalyzed by different aromatic hydrocarbon dioxygenases suggests that indole may be a valuable substrate for elucidating the mechanism of action of this class of enzymes.

Note added in proof: Another method for the cloning and expression of naphthalene oxidation genes in *E. coli* has been reported by Schell (*17*).

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Anticarcinoma Activity in vivo of Rhodamine 123, a Mitochondrial-Specific Dye

Abstract. *Carcinoma cells and normal epithelial cells differ in the mitochondrial retention of a permeant cationic compound, rhodamine 123. The possibility of utilizing this difference in carcinoma chemotherapy was investigated. Rhodamine 123 exhibited anticarcinoma activity in mice, and this activity was potentiated by 2-deoxyglucose.*

Epithelial cancers, particularly those of the breast and colon, are the major causes of death due to cancer in the United States. Most of the anticarcinoma drugs in clinical use are targeted at the DNA of the cell. Carcinoma chemotherapy in which drugs are targeted at the plasma membrane, mitochondria, endoplasmic reticulum, cytoskeleton, or intermediary metabolism unrelated to DNA is largely unexplored. We discovered that a fluorescent dye, rhodamine 123 (Rh123), localizes in the mitochondria of living cells (*1*), probably as a result of high membrane potential across the mitochondrial membrane (*2*). The mitochondria of a variety of carcinomas retain Rh123 for prolonged periods (2 to 5 days), whereas normal epithelial cells release it within a few hours (*3*). This unexpected finding prompted us to investigate whether this difference in the mitochondria of carcinoma and normal epithelial cells can be utilized in cancer chemotherapy. We reported earlier that Rh123 is selectively toxic to carcinoma cells in vitro (*4, 5*). We now report that Rh123 has anticarcinoma activity in mice and that this activity is potentiated by 2-deoxyglucose, an inhibitor of glycolysis.

Ehrlich ascites tumor cells, confirmed to be of epithelial origin by immunofluorescence with antibody to keratin, were injected intraperitoneally (5×10^5 cells) into mice. These tumor-bearing mice had a narrow range of survival times (18 to 22 days; median, 19 days), reflecting consistency in the mortality pattern (Fig. 1

Table 1. Survival of mice bearing Ehrlich ascites tumors treated with rhodamine 123 (Rh123) 2-deoxyglucose (2-DG), or both. Ehrlich ascites tumor cells (5×10^5) were injected intraperitoneally into BDF1 female mice aged 5 to 6 weeks. Intraperitoneal drug injections were started 24 hours later (day 1). Survival was expressed as T/C (median survival time of drug-treated mice as a percentage of the median survival time of untreated controls). Ten mice used as untreated controls had a median survival of 19 days; seven mice were used in each drug treatment group. The results shown are averages of five different experiments. Results shown in parentheses are from individual experiments. Arabinosylcytosine (Ara-C) was used as a positive control.

Treatment	T/C (%)
Rh123 (5 mg/kg, days 1, 3, 5, 7, 9)	105
Rh123 (10 mg/kg, days 1, 3, 5, 7, 9)	115
Rh123 (10 mg/kg, days 1 to 10)	120
Rh123 (15 mg/kg, days 1, 3, 5)	260 (253, 258, 263, 263, 273)
Rh123 (15 mg/kg, days 1 to 5)	70
Rh123 (20 mg/kg, days 1, 3, 5)	121
2-DG (0.5 g/kg, days 1, 3, 5)	100 (100, 105, 100, 100, 100)
Rh123 (15 mg/kg, days 1, 3, 5) plus 2-DG (0.5 g/kg, days 1, 3, 5)	420 (390, 390, 400, 521, 420)
Ara-C (200 mg/kg, days 1 to 5)	150

Table 2. Survival of mice bearing MB49 bladder carcinoma treated with rhodamine 123 (Rh123), 2-deoxyglucose (2-DG), or both. MB49 (1×10^6 cells) were injected intraperitoneally in BDF1 mice. Drug treatments were started 24 hours later. Ten mice used as untreated controls had a median survival of 19 days; seven mice were used in each drug treatment group. The results shown are averages of five separate experiments. Results of some individual experiments are shown in parentheses. Arabinosylcytosine (Ara-C) was used as a positive control.

Treatment	T/C (%)
Rh123 (5 mg/kg, days 1, 3, 5, 7, 9)	110
Rh123 (10 mg/kg, days 1, 3, 5, 7, 9)	115
Rh123 (10 mg/kg, days 1 to 10)	120
Rh123 (15 mg/kg, days 1, 3, 5)	180 (157, 179, 184, 184, 189)
Rh123 (15 mg/kg, days 1 to 5)	110
Rh123 (20 mg/kg, days 1, 3, 5)	90
2-DG (0.5 g/kg, days 1, 3, 5)	100 (98, 105, 100, 100, 105, 105)
Rh123 (15 mg/kg, days 1, 3, 5) plus 2-DG (0.5 g/kg, days 1, 3, 5)	250 (188, 252, 305, 263, 252)
Ara-C (200 mg/kg, days 1 to 5)	150

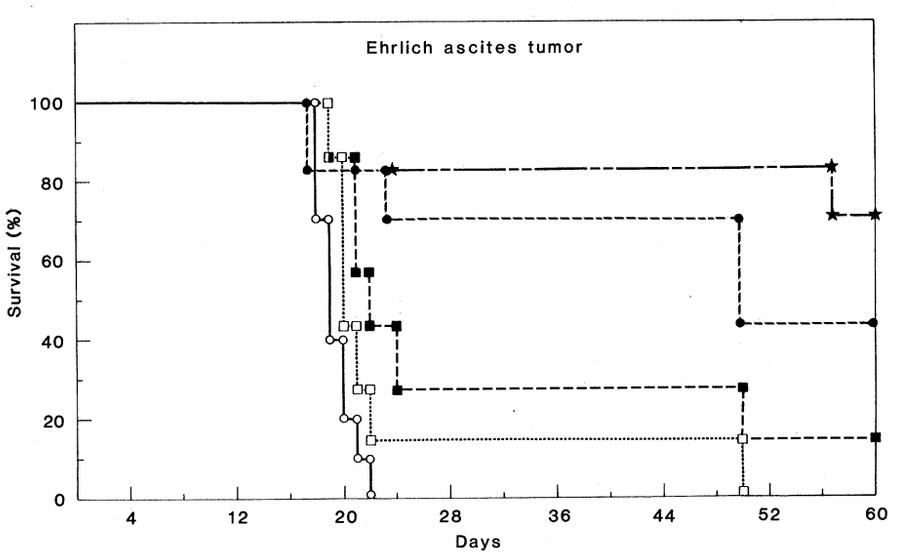

Fig. 1. Effect of rhodamine 123 and 2-deoxyglucose on survival of mice implanted with Ehrlich ascites carcinoma: (○) Untreated controls; T/C, 100 percent. (□) Rh123 (5 mg/kg, days 1, 3, 5, 7, 9); T/C, 105 percent. (■) Rh123 (10 mg/kg, days 1, 3, 5, 7, 9); T/C, 115 percent. (●) Rh123 (15 mg/kg, days 1, 3, 5); T/C, 263 percent. (▲) Rh123 (15 mg/kg, days 1, 3, 5) plus 2-DG (0.5 g/kg, days 1, 3, 5); T/C, 400 percent. Each experiment included ten mice as untreated controls and seven mice in each drug treatment group.

and Table 1). Treatment with Rh123 (5 to 10 mg/kg) on days 1, 3, 5, 7, and 9 or on days 1 to 10, did not significantly prolong survival of carcinoma-bearing mice; the median survival time was 20 to 21 days, and T/C (median survival time of drug-treated mice as a percentage of the median survival time of untreated controls) was 110 to 120 percent. When the maximum nonlethal dose of Rh123 was used (15 mg/kg on days 1, 3, and 5), survival was prolonged to a median of 50 days (T/C, 260 percent). Approximately 10 percent of the mice showed no evidence of tumor when they were killed 90 days after tumor implantation. Other schedules of treatment with large doses of Rh123, such as 15 mg/kg on days 1 to 5 or 20 mg/kg on days 1, 3, and 5, were ineffective in prolonging survival because of early deaths (on days 5 to 9) attributable to drug toxicity. Arabinosylcytosine (Ara-C) (200 mg/kg on days 1 to 5), used as a positive control, prolonged survival to 29 days (T/C, 150 percent).

Rhodamine 123 accumulates selectively in mitochondria and indirectly inhibits mitochondrial adenosine triphosphate (ATP) synthesis (*6–8*). 2-Deoxyglucose inhibits glycolysis, the pathway for nonmitochondrial ATP production. It was of interest, therefore, to determine whether 2-deoxyglucose would potentiate the anticarcinoma activity of Rh123. 2-Deoxyglucose (0.5 g/kg on days 1, 3, and 5) alone had no effect on survival of tumor-bearing mice (T/C, 100 percent). However, treatment with the combination of 2-deoxyglucose (0.5 g/kg on days 1, 3, and 5) and Rh123 (15 mg/kg on days 1, 3, and 5) prolonged median survival to 80 days (T/C, 420 percent). Approximately 40 percent of the mice treated with this combination had no evidence of tumor at 90 days.

Mice injected intraperitoneally with 1×10^6 MB49 cells, a mouse bladder cell line induced by 7,12-dimethylbenz(a)nthracene (*9*), died between 17 and 22 days (median, 19 days). MB49-bearing mice treated with Rh123 at 5 to 10 mg/kg administered intraperitoneally on days 1, 3, 5, 7, and 9 or on days 1 to 10 had a median survival time of 21 to 23 days (T/C, 110 to 120 percent). (Fig. 2 and Table 2). However, survival was prolonged to 34 days (T/C, 180 percent) with Rh123 administered intraperitoneally at 15 mg/kg on days 1, 3, and 5. As with the Ehrlich ascites tumor-bearing mice, median survival was shorter when other schedules of Rh123 were used (for example, 15 mg/kg on days 1 to 5 or 20 mg/kg on days 1, 3, and 5. 2-Deoxyglucose alone did not prolong the survival of

MB49-bearing mice. The combination of 2-deoxyglucose (0.5 g/kg on days 1, 3, and 5) and Rh123 (15 mg/kg on days 1, 3, and 5) prolonged survival to 48 days (T/C, 250 percent). Approximately 10 percent of these mice showed no evidence of their tumor at 90 days. Higher doses of 2-deoxyglucose (1 g/kg on days 1, 3, and 5) used in combination with Rh123 resulted in early deaths due to drug toxicity, although this higher dose of 2-deoxyglucose by itself was not lethal. Ara-C (200 mg/kg on days 1 to 5), used as a positive control, prolonged survival to 29 days (T/C, 150 percent). Leukemia and melanoma cell lines, unlike carcinomas, release Rh123 within 16 to 24 hours in rhodamine-free medium (*3*). Lewis lung carcinoma cells, like those of human oat cell carcinoma, contain keratin but are not long retainers of Rh123 in vitro. Because L1210 leukemia, P388 leukemia, B16 melanoma, and Lewis lung carcinoma are commonly used to screen potential anticancer drugs, we tested the antitumor activity of Rh123 in these tumor models. As shown in Table 3, Rh123 did not significantly prolong survival of mice implanted with these tumors.

We have found that Rh123 prolonged survival of mice implanted with Ehrlich ascites tumor or MB49 mouse bladder carcinoma. The prolongation of survival was dependent on the dose and schedule of administration. The anticarcinoma activity of Rh123 was most marked when administered at the highest tolerable doses soon after tumor implantation (15 mg/kg on days 1, 3, and 5). Other maximum nonlethal dose schedules, such as 10 mg/kg on days 1 to 10 and 5 mg/kg on days 1 to 12, were not as effective, although the cumulative doses were at least as high as those attained with treatment on days 1, 3, and 5. Thus, pulse treatment with high doses of Rh123 appears to be more effective than prolonged administration of small doses. Pulse treatment with large doses of Rh123 may have led to selective retention of high concentrations of Rh123 in carcinoma cells. We observed earlier that carcinomas retained Rh123 for long periods (2 to 5 days) after a short pulse of Rh123 treatment in vitro, whereas normal epithelial cells, hematopoietic cells, and fibroblasts released the dye within 1 to 16 hours in dye-free medium (*3*). Cells that retained Rh123 for long periods seemed to be more sensitive to the toxic effect of Rh123 in vitro than cells that retained Rh123 for short periods (*4, 5*). Prolonged retention of dye by carcinomas may be responsible also for the anticarcinoma activity of Rh123 in vivo. Support of this hypothesis is provided by the absence of antitumor activity of Rh123 in mice implanted with L1210 leukemia, P388 leukemia, Lewis lung carcinoma, and B16 melanoma, which retain Rh123 for short periods in vitro.

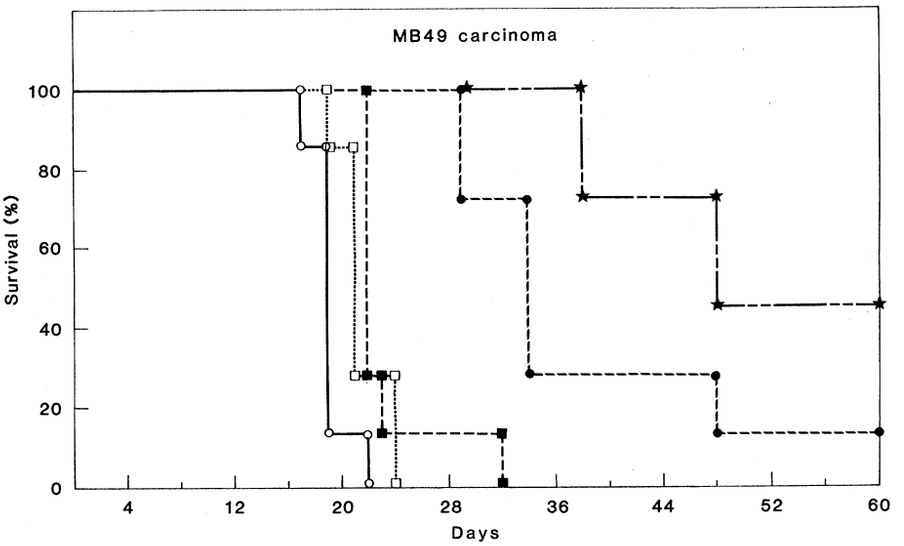

Fig. 2. Effect of rhodamine 123 and 2-deoxyglucose on survival of mice implanted with MB49 carcinoma cells: (○) Untreated controls; T/C, 100 percent. (□) Rh123 (5 mg/kg, days 1, 3, 5, 7, 9); T/C, 110 percent. (■) Rh123 (10 mg/kg, days 1, 3, 5, 7, 9); T/C, 115 percent. (●) Rh123 (15 mg/kg, days 1, 3, 5); T/C, 179 percent. (▲) Rh123 (15 mg/kg, days 1, 3, 5) plus 2-DG (0.5 g/kg, days 1, 3, 5); T/C, 263 percent. Each experiment included ten mice as untreated controls and seven mice in drug treatment groups.

Table 3. Survival of mice with various tumors or leukemias after treatment with rhodamine 123 (Rh123). L1210 leukemia cells (10^5), P388 leukemia cells (10^6), or B16 melanoma cells (10^7) were injected intraperitoneally, and Lewis lung carcinoma cells (2×10^6) were injected intramuscularly into BDF1 mice. Drugs were injected intraperitoneally starting at 24 hours after tumor implantation. Ten mice were used as untreated controls; seven mice were used for each drug treatment group.

Treatment	T/C (%)
L1210 leukemia	
Rh123 (10 mg/kg, days 1 to 10)	110
Rh123 (10 mg/kg, days 1, 3, 5, 7, 9)	110
Rh123 (15 mg/kg, days 1, 3, 5)	110
Rh123 (20 mg/kg, days 1, 3, 5)	110
P388 leukemia	
Rh123 (10 mg/kg, days 1 to 10)	110
Rh123 (10 mg/kg, days 1, 3, 5, 7, 9)	110
Rh123 (15 mg/kg, days 1, 3, 5)	110
Rh123 (20 mg/kg, days 1, 3, 5)	100
B16 melanoma	
Rh123 (10 mg/kg, days 1 to 10)	115
Rh123 (10 mg/kg, days 1, 3, 5, 7, 9)	110
Rh123 (15 mg/kg, days 1, 3, 5)	100
Rh123 (20 mg/kg, days 1, 3, 5)	100
Lewis lung carcinoma	
Rh123 (5 mg/kg, days 1 to 10)	110
Rh123 (10 mg/kg, days 1 to 10)	100
Rh123 (20 mg/kg, days 1, 3, 5)	100

Mitochondria are the likely initial targets for the cytotoxic action of Rh123 since the dye is selectively accumulated by mitochondria (*1–3*). Rhodamine 123 disrupts mitochondrial functions such as ATP synthesis (*6–8*). The anticarcinoma activity of Rh123 was markedly potentiated by 2-deoxyglucose, although 2-deoxyglucose by itself did not prolong survival of tumor-bearing mice. The simultaneous inhibition of ATP synthesis from mitochondrial metabolism and glycolysis may provide a rationale for the combined use of Rh123 and 2-deoxyglucose. There is a particular need to develop new and more selective drugs against epithelial tumors, especially those originating in the lung, breast, and colon (*10*). Our results may be the basis for clinical application of Rh123, as well as for a screening program to search for permeant cationic compounds more effective than Rh123.

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22 August 1983

Potentiation of Glucocorticoid Activity by 5β-Dihydrocortisol: Its Role in Glaucoma

Abstract. *5β-Dihydrocortisol potentiated the threshold level (the smallest dose producing a measurable effect) of topically applied cortisol (0.02 percent) and dexamethasone (0.003 percent) in causing nuclear translocation of the cytosolic glucocorticoid receptor in rabbit iris–ciliary body tissue. 5β-Dihydrocortisol accumulates in cells cultured from trabecular meshwork specimens from patients with primary open-angle glaucoma, but not in similar cells derived from nonglaucomatous patients. In view of the sensitivity of patients with primary open-angle glaucoma to the effects of glucocorticoids in raising intraocular pressure, this potentiation may be responsible for the steroid sensitivity and for the ocular hypertension seen in this disorder.*

Primary open-angle glaucoma (POAG) is the most common form of glaucoma and a major cause of blindness. Most patients with this disorder show a marked sensitivity to the effects of topical and systemic glucocorticoids in raising intraocular pressure (*1*). This suggests that endogenous cortisol plays a role in the ocular hypertension found in POAG.

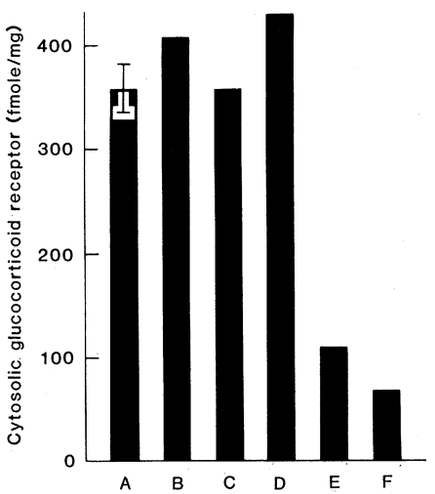

Fig. 1. Glucocorticoid receptor concentration in the cytosol of rabbit iris–ciliary body after topical (ocular) administration of (A) vehicle (PBS), (B) 1 percent 5β-tetrahydrocortisol, (C) 1 percent 5α-dihydrocortisol, (D) 1 percent 5β-dihydrocortisol, (E) 1 percent cortisol, and (F) 0.1 percent dexamethasone-21-phosphate. The values shown represent the averages of at least two separate experiments. In each experiment, duplicate measurements were made on pooled tissue from at least three animals. In (A) the standard deviation was determined from seven separate experiments and is indicated on the bar graph. The animals were killed 1 hour after topical application of 25 μl of the indicated steroid solution. When there was no response (A through D), two applications of test solutions were used, at 2 hours and at 1 hour before the animal was killed.

We reported earlier that cortisol metabolism is altered in cells cultured from specimens obtained by trabeculectomy from patients with POAG (*2*). These cells accumulated 5β-dihydrocortisol and, to a lesser extent, 5α-dihydrocortisol—intermediates not found in similar cells derived from non-POAG patients. In view of the sensitivity of POAG patients to the effect of glucocorticoids in raising intraocular pressure, we undertook a study of the biologic activity of dihydrocortisol. Nuclear translocation of the cytoplasmic glucocorticoid receptor in rabbit iris–ciliary body tissue was used as a test system. Specific glucocorticoid target cells have been identified in the outflow pathway region of the rabbit by both biochemical and autoradiographic techniques (*3*). After topical and systemic administration of active glucocorticoids, there was a loss of glucocorticoid receptors from the cytoplasm with a concomitant gain in the nucleus (*4*). The median effective dose of various steroids for translocation of the glucocorticoid receptor to the nucleus was found to be correlated with the activity of the steroids in raising intraocular pressure (*4*). We now report that 5β-dihydrocortisol potentiates the cortisol- and dexamethasone-induced translocation of the cytoplasmic glucocorticoid receptor to the nucleus. This translocation is an early and necessary event in the action of steroid hormones.

Young New Zealand rabbits weighing less than 2 kg were used as the experimental animals. A control group and an experimental group of three animals each were used in each experiment. The steroids were suspended in phosphate-buffered saline (PBS) by homogenization with a Teflon pestle; this produced a fine suspension of the steroids that minimized corneal irritation. One hour after the last steroid treatment the animals were killed, and the iris–ciliary body was removed, homogenized, and assayed for cytosolic glucocorticoid receptor concentration by determining the specific binding of [^{3}H]dexamethasone (*4*). The concentration of labeled dexamethasone and the conditions of incubation were sufficient to saturate unbound receptors and to exchange (more than 90 percent) with receptor-bound nonlabeled cortisol or corticosterone (*4*).

Figure 1 shows the amount of receptor remaining in the cytoplasm after treatment with different steroids (*5*). Animals

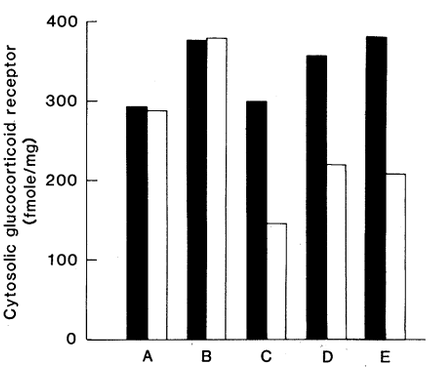

Fig. 2. Potentiation of glucocorticoid activity by cortisol metabolites. Glucocorticoid receptor concentration in cytosol was measured after topical (ocular) administration of threshold levels of cortisol (0.02 percent) and dexamethasone-21-phosphate (0.003 percent), with and without steroid metabolites. The values shown represent the average of at least two separate experiments, with six animals used for each (three control and three experimental). Paired groups of animals were used to control for possible variations due to different batches of animals and day-to-day biologic fluctuations. One group of three animals each received threshold levels of glucocorticoids alone (solid bars): (A through D) cortisol and (E) dexamethasone-21-phosphate. The other group of three animals each received threshold levels of one of these glucocorticoids with the test metabolites (open bars). The test metabolite was given 2 hours and again 1 hour before the animal was killed. The threshold level of glucocorticoid was administered 1 hour before the animal was killed. The test metabolites and threshold glucocorticoids (in parentheses) were (A) 1 percent 5β-tetrahydrocortisol (cortisol); (B) 1 percent 5α-dihydrocortisol (cortisol); (C) 1 percent 5β-dihydrocortisol (cortisol); (D) 0.1 percent 5β-dihydrocortisol (cortisol); and (E) 1 percent 5β-dihydrocortisol (dexamethasone-21-phosphate).