ing water movement to growth in branched scleractinian corals, octocorals, poriferans, and hydrozoans (*18, 19*). Computer simulation allows investigators to alter each morphological parameter independently or in combination with others, thereby permitting exploration of the exact nature and limits of the adaptive mechanisms. Computer output in the form of maps or serial sections could serve as plans for constructing physical models (*19*). Such models could then be tested experimentally in a flume with flow measuring equipment (*18*), and the results could be compared with the actual structures of corals living in known energy regimes on a reef.

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Calcium-Independent Modulation of Cyclic GMP and Activation of Guanylate Cyclase by Nitrosamines

Abstract. *Nitrosamines markedly increase concentrations of guanosine 3',5'-monophosphate (cyclic GMP) in several tissues from the rat and in human colonic mucosa. These agents are effective in the absence of extracellular calcium and enhance guanylate cyclase activity in tissue homogenates. Stimulation of cyclic GMP was greatest in liver, where the carcinogenic activity of nitrosamines is also most pronounced.*

There is evidence to suggest that guanosine 3',5'-monophosphate (cyclic GMP) may promote cell growth and transformation (*1*), although not all data are consistent with this hypothesis (*2*). In the study reported here, we demonstrate that nitrosamines, compounds with well-established carcinogenic and mutagenic properties which may be formed in the digestive tract from dietary constituents and drugs (*3–5*), are potent agonists of guanylate cyclase activity in tissue homogenates and of cyclic GMP accumulation in tissue slices. The action of nitrosamines to increase cellular cyclic GMP concentrations can be fully expressed in the absence of extracellular Ca^{2+}. The Ca^{2+}-independent action of nitrosamines on cyclic GMP and their ability to activate guanylate cyclase in tissue homogenates resemble the recently described properties of sodium azide (*6–8*), but differ from those of most of the currently recognized physiologic agonists of cyclic GMP, such as cholinergic and α-adrenergic stimuli. These latter agents both require Ca^{2+} to increase cellular cyclic GMP accumulation and fail to activate guanylate cyclase in broken cell preparations (*9, 10*). With nitrosamines, enhancement of guanylate cyclase activity and cyclic GMP accumulation are greatest in liver, where their carcinogenic action is also most pronounced (*5*). The effects of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), a potent carcinogen with a considerable propensity toward free radical formation (*11*), are particularly striking. Indeed, MNNG may be the most potent agonist of guanylate cyclase activity yet identified.

Slices of liver, renal cortex, and lung were prepared with a Stadie-Riggs microtome, and scrapings of colonic mucosa were obtained with a glass slide. Organs were excised from 300-g male Sprague-Dawley rats fasted 18 hours and then anesthetized with pentobarbital. Human colonic mucosa was obtained from patients undergoing therapeutic segmental resections for carcinoma or diverticular disease. To assay cyclic GMP content, tissue was initially incubated for 20 minutes at 37°C in 2 ml of Krebs-Ringer bicarbonate buffer (Ca^{2+}, 1.5 m*M*) with glucose and albumin (1 mg/ml) in either the absence or presence of 2 m*M* 1-methyl-3-isobutyl-xanthine (MIX) to inhibit cyclic nucleotide breakdown. Test agents were then added and incubations continued for the times indicated. Incubations were terminated by homogenizing tissue in 5 percent trichloroacetic acid at 0° to 4°C. Cyclic GMP was then extracted and measured by radioimmunoassay, as previously reported (*12*), while the cyclic adenosine monophosphate (AMP) content of the same extracts was determined by the protein-binding method (*12*). To assay guanylate cyclase activity, tissue was gently homogenized in a solution containing 0.25*M* sucrose and 5 m*M* tris buffer, *p*H 7.4 (25 mg/ml). Appropriate dilutions of whole homogenates or the 100,000*g* soluble and particulate fractions were employed. Activity was determined from the conversion of [α-^{32}P]guanosine triphosphate (GTP) to cyclic GMP, using a reaction mixture (1 m*M* GTP, 4 m*M* Mn^{2+}, creatine phosphate–creatine phosphokinase GTP regenerating system) and a two-step chromatographic technique (AG 50 WX4 followed by neutral alumina) for purification of cyclic GMP that have been described in detail (*13*). Reactions were conducted at 37°C for 7 minutes. Cyclic GMP formation was linear with time for at least 10 minutes and with respect to added protein. Dithiothreitol (2.5 m*M*) was included in homogenates and reaction mixtures of lung and colonic mucosa to prevent "autoactivation" of the enzyme (*14*) observed in these two tissues. The MIX and MNNG were obtained from Aldrich Chemical Co., Milwaukee, Wis.; diethylnitrosamine (DEN), dibutylnitrosamine (DBN), nitrosopiperidine (NP), hydroxylamine (NH_2OH), and sodium nitrite were purchased from Fisher Scientific Co., Pittsburgh, Pa. Sources of all other reagents have been reported (*7, 12, 13, 15*). Each experiment was repeated at least twice.

As shown in Table 1, MNNG, DEN, DBN, and NP increased hepatic cyclic GMP 4- to 33-fold in 2 minutes. With MNNG, increases in hepatic cyclic GMP were detected within 15 seconds (3-fold) and levels remained elevated for at least

60 minutes (16-fold). When examined at time intervals ranging from 2 to 30 minutes, at the concentrations shown in Table 1, the relative potency of the four nitrosamines as agonists of cyclic GMP in hepatic slices remained constant (MNNG > NP > DEN > DBN). Thus, MNNG was by far the most effective nitrosamine tested, when cyclic GMP was determined at 5, 10, 20, and 30 as well as 2 minutes after the addition of these agents. In liver, minimal effective concentrations were 1 μM for MNNG and 10 μM for DEN, DBN, and NP, when responses were examined at 2 minutes. Alterations in hepatic cyclic GMP content in response to nitrosamines did not depend on the presence of MIX in the incubation media, although the latter agent potentiated the magnitude of the increases observed. Thus, without an inhibitor of phosphodiesterase activity, hepatic cyclic GMP (basal, 0.21 ± 0.02 pmole per milligram of protein) rose 22-fold with MNNG, 7-fold with DEN, 2.5-fold with DBN, and 10-fold with NP in 2 minutes. The concentrations of the nitrosamines that were maximally effective in the liver also significantly increased cyclic GMP in renal cortex, lung, and colonic mucosa from the rat and in human colonic mucosa (Table 1). Although these tissues were less responsive than liver, cyclic GMP rose 12- to 21-fold in 2 minutes with MNNG. As also indicated in Table 1, NH_2OH (10 mM) and nitrite (20 mM) increased cyclic GMP in all tissues studied, while neither these agents nor the nitrosamines significantly altered tissue cyclic AMP content (not shown).

The incubation of hepatic or renal cortical slices in Ca^{2+}-free buffer for 20 minutes reduced basal cyclic GMP levels, but either did not alter or enhanced the relative cyclic GMP responses of these tissues to the nitrosamines, NH_2OH, or nitrite (Table 2). By contrast, exclusion of Ca^{2+} has been shown to abolish completely the action of cholinergic and other physiologic agonists to increase cellular cyclic GMP levels (*9, 15*). As also shown in Table 2, the nitrosamines, NH_2OH (*16*), and nitrite (*16*) significantly enhanced guanylate cyclase activity in whole homogenates of liver and renal cortex. In agreement with changes in tissue cyclic GMP levels, increases in enzyme activity were greatest with MNNG (36-fold in liver). Similarly, when tested in rat lung and in rat or human colonic mucosa at the concentrations indicated in Table 1, the nitrosamines, NH_2OH, and nitrite activated guanylate cyclase in tissue homogenates and significantly increased cellular cyclic GMP in the absence of Ca^{2+} (not shown). Stimulation of guanylate cyclase activity by the nitrosamines was comparable in whole homogenates of liver and renal cortex and in the 100,000g soluble fractions from these tissues, whereas particulate enzyme activity from these tissues responded only to MNNG (2-fold increase). The mechanism by which nitrosamines activate guanylate cyclase is unknown. These agents, NH_2OH, and nitrite all form free radicals in tissue (*11, 17–19*). Of the agents tested to date, MNNG is the most potent agonist of guanylate cyclase and also demonstrates the greatest propensity toward formation of free radicals (*11*). The latter have been shown to interact with both nucleic acids and proteins (*18*). Thus, free radical formation by these agents and their activation of guanylate cyclase may be linked.

It is of some interest that enhancement of guanylate cyclase activity and tissue cyclic GMP levels by nitrosamines is greatest in liver, where the carcinogenicity of these compounds is also most evident (*8*). Our studies do not establish a causal relationship between the ability of nitrosamines to increase guanylate cyclase activity or tissue cyclic GMP content and their carcinogenic properties. However, cyclic GMP has been reported to enhance cell growth (*1, 20*), and increased levels of this nucleotide occur in some malignancies (*12, 21*). Further, the mechanism by which nitrosamines increase cyclic GMP is clearly distinct from that of many physiologic agonists, which are both ineffective in the absence of extracellular Ca^{2+} and fail to increase guanylate cyclase when added directly to tissue homogenates. Accordingly, it is possible that local cellular feedback control mechanisms available to modulate cyclic GMP accumulation in response to Ca^{2+}-dependent, physiologic agonists of the nucleotide may be circumvented by the nitrosamines. The extent to which other chemical carcinogens similarly acti-

Table 1. Effects of nitrosamines, NH_2OH, and $NaNO_2$ on tissue cyclic GMP content in the presence of Ca^{2+}. Tissues were initially incubated in Krebs-Ringer bicarbonate buffer containing 2 mM MIX for 20 minutes at 37°C with test agents present for the final 2 minutes. The MNNG and DBN were dissolved in acetone, which alone had no effect on cyclic GMP. Each value represents the mean ± standard error (S.E.) for six slices of each tissue from the same experiment. Concentrations in column 1 are the maximally effective concentrations in liver.

Addition	Cyclic GMP content (picomoles per milligram of protein)				
	Rat liver	Rat renal cortex	Rat lung	Rat colonic mucosa	Human colonic mucosa
None	0.36 ± 0.04	1.05 ± 0.09	2.74 ± 0.31	1.29 ± 0.13	0.78 ± 0.08
MNNG (1 mM)	11.77 ± 1.08*	18.36 ± 2.10*	34.28 ± 3.56*	27.53 ± 2.60*	9.31 ± 1.06*
DEN (20 mM)	3.86 ± 0.45*	2.27 ± 0.25*	4.62 ± 0.52†	2.32 ± 0.26†	1.53 ± 0.18†
DBN (20 mM)	1.40 ± 0.21*	1.94 ± 0.21†	4.11 ± 0.45†	2.03 ± 0.22†	1.45 ± 0.17†
NP (20 mM)	5.42 ± 0.65*	3.18 ± 0.36*	4.91 ± 0.48†	2.57 ± 0.31†	1.68 ± 0.21†
NH_2OH (10 mM)‡	7.58 ± 0.86*	11.40 ± 1.28*	14.70 ± 1.55*	14.82 ± 1.53*	5.52 ± 0.64*
$NaNO_2$ (20 mM)	4.93 ± 0.52*	6.32 ± 0.71*	11.28 ± 0.96*	12.36 ± 0.45*	2.60 ± 0.30†

*$P < .001$ compared to the corresponding control level by unpaired t-test. †$P < .025$ compared to control. ‡The pH was adjusted to 7.0 with 2M tris base before addition.

Table 2. Effects of nitrosamines, NH_2OH, and $NaNO_2$ on cyclic GMP contents of hepatic and renal cortical slices in Ca^{2+}-free Krebs-Ringer bicarbonate buffer and on guanylate cyclase activity of whole tissue homogenates. Tissue slices were incubated in buffer containing 0.5 mM EGTA and 2 mM MIX for 20 minutes, with test agents present for the final 2 minutes. Guanylate cyclase reactions were conducted for 7 minutes at 37°C. Preincubation of tissue homogenate with test agents for 5 minutes did not significantly enhance enzyme activation. Abbreviations are given in the text; concentrations of test agents are as shown in Table 1. Each value for cyclic GMP represents the mean ± S.E. for six slices of each tissue from the same experiment. Guanylate cyclase activities (picomoles of cyclic GMP per minute per milligram of protein) are means of duplicate determinations from three separate experiments.

Addition	Cyclic GMP content (picomoles per milligram of protein)		Guanylate cyclase activity	
	Liver	Renal cortex	Liver	Renal cortex
None	0.19 ± 0.02	0.43 ± 0.04	24 ± 2	51 ± 2
MNNG	7.03 ± 0.74*	20.67 ± 2.29*	866 ± 60*	968 ± 93*
DEN	2.16 ± 0.25*	1.05 ± 0.11†	45 ± 3†	116 ± 7*
DBN	0.64 ± 0.06*	0.98 ± 0.09†	44 ± 3†	98 ± 10†
NP	2.27 ± 0.26*	1.41 ± 0.18†	61 ± 5*	163 ± 19†
NH_2OH	4.56 ± 0.51*	12.38 ± 1.37*	240 ± 19*	728 ± 61*
$NaNO_2$	2.19 ± 0.23*	2.26 ± 0.34*	441 ± 32*	915 ± 98*

*$P < .001$ compared to corresponding control level. †$P < .025$ compared to control.

vate guanylate cyclase and increase tissue cyclic GMP is of obvious interest. Goldberg *et al.* (*20*) reported that phorbal myristate acetate, the biologically active component of croton oil, increases cyclic GMP in cultured fibroblasts, but data are otherwise lacking. Conversely, questions arise concerning the mutagenic and carcinogenic potential of other agonists of guanylate cyclase, such as NH_2OH and nitrite, whose effects on the enzyme appear quite similar to those of the nitrosamines. While administration of nitrite to intact animals has failed to induce tumors (*19, 22*), this agent has been reported to transform cells in culture (*23*). With regard to the action of NH_2OH on guanylate cyclase, it is pertinent to note that the carcinogenic activity of some aromatic amines depends on their metabolism to *N*-hydroxylamine derivatives and that NH_2OH is mutagenic (*24, 25*). Further examination of the relationship between chemical carcinogenesis and the guanylate cyclase–cyclic GMP system is indicated.

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Diamond-Blackfan Syndrome: Lymphocyte-Mediated Suppression of Erythropoiesis

Abstract. *Peripheral blood lymphocytes from six patients with congenital hypoplastic anemia suppressed erythroid cell formation by normal human bone marrow cells in response to erythropoietin in vitro. The results suggest that the anemia in these children has an immunologic basis.*

Congenital hypoplastic anemia (CHA, or Diamond-Blackfan syndrome) is a disorder of childhood; it is characterized by progressive anemia beginning early in infancy, and is sparing of the white blood cells and platelets (*1*). In the bone marrow, erythroid precursors are virtually absent. Since its initial description in 1938 (*2*), the etiology of CHA has remained unknown. The production of erythropoietin (Ep) (*3*), and the numbers of erythroid progenitor cells (*4*) capable of proliferation and differentiation into red cells appear to be adequate. In contrast to pure red cell aplasia (PRCA) acquired by adults (*5*), a serum inhibitor of erythropoiesis is not present in CHA (*6*). Peripheral blood lymphocytes from an adult with common variable hypogammaglobulinemia and PRCA can inhibit erythropoiesis in vitro (*7*). We now present evidence that peripheral blood lymphocytes from patients with CHA inhibit red cell production by normal bone marrow cells, an indication that CHA may have an immunologic basis.

Peripheral blood lymphocytes from six patients with clinically established CHA were separated from heparinized whole blood by means of a Ficoll-Hypaque density gradient (*8*). Cells from 24 randomly selected normal subjects, five individuals who were multiply transfused with blood products for a variety of other disorders, and three adults with acquired PRCA, were used in this study. The control group included individuals matched as to sex and age to the patients with CHA. Control studies were performed in parallel with experimental studies. Normal human bone marrow cells were obtained by aspiration from the posterior iliac crest of 20 individuals with a normal hematological profile. Varying numbers of peripheral blood lymphocytes (0.5×10^5, 1×10^5, 2×10^5, and 4×10^5 cells) were cultured with 6×10^5 bone marrow cells in the plasma clot culture system (*9*) in

Table 1. Effect of human peripheral blood lymphocytes on erythroid colony formation by normal human bone marrow cells in response to erythropoietin in vitro. Each value represents the mean ± 1 standard error of the mean of results obtained from all studies. The numbers in parentheses indicate the number of studies conducted.

Additions to culture	Number of erythroid colonies per 6×10^5 bone marrow cells at lymphocyte concentrations of			
	0.5×10^5	1×10^5	2×10^5	4×10^5
None	0	0	0	0
Normal lymphocytes	0	0	0	0
2 I.U. of Ep*		*422 ± 21*		
+ Normal lymphocytes (24)	576 ± 18	612 ± 32	594 ± 17	572 ± 26
+ Lymphocytes, transfused patients (5)	632 ± 68	608 ± 72	576 ± 53	575 ± 49
+ PRCA lymphocytes (3)	486 ± 47	509 ± 55	617 ± 89	603 ± 77
+ CHA lymphocytes (12)	46 ± 9	12 ± 6	22 ± 7	110 ± 15
+ F-T CHA lymphocytes†	‡	‡	494 ± 72	‡

*Human urinary erythropoietin used in these studies was collected and concentrated by the Department of Physiology, University of Northeast, Corrientes, Argentina, and further processed and assayed by the Hematology Research Laboratories, Children's Hospital of Los Angeles, under NIH grant HE-10880. †Lymphocytes were subjected to repeated freeze-thawing (F-T) prior to use. ‡Not determined.