up amine precursors and modify them by decarboxylation. Although these cells are not all embryologically derived from the neural crest (*10*), they contain the neuroendocrine marker, neuron-specific enolase (*11*). Not all cells of the DNE system or other cell types with granules are reactive with LK2H10 at the concentrations we used: pancreatic exocrine cells and neurons from the hypothalamus and frontal lobes of the brain and nerve terminals in the posterior pituitary were all negative, as were melanomas. It is not clear whether these cells contain too few granules to exhibit staining or whether they simply do not synthesize the 68,000-dalton substance identified by LK2H10. However, this lack of reactivity with neural tissues distinguishes LK2H10 from previously reported monoclonal antibodies that identify a structure of similar molecular weight in secretory vesicles of both endocrine and neural tissues (*4*). The ability of LK2H10 to react with a specific subset of tissues of the DNE system may be advantageous for analyzing subgroups of DNE system cells, since other neuroendocrine markers such as enolase are found in all of the cells of this system.

In a recent study O'Connor *et al.* (*12*) detected human chromogranin A by immunohistochemistry in eight polypeptide-producing endocrine tumors by using a polyclonal antiserum. Since the molecular weights of human chromogranin A and the major fraction of our pheochromocytoma antigen are both 68,000, monoclonal antibody LK2H10 may be directed against a chromogranin A–like substance.

The availability of large quantities of this monoclonal antibody, which is restricted in specificity to endocrine cells with secretory granules, should permit detailed analyses of normal tissues and tumors of the DNE system from various perspectives. First, it may be possible to analyze the distribution of this antigen in the serum as well as in tumors in patients with neuroendocrine tumors. This antigen can also be used as a marker in Formalin-fixed, paraffin-embedded tissues to diagnose endocrine tumors that have dense-core granules. Second, the development and distribution of neuroendocrine tissues in human fetuses can be analyzed with this marker, especially as they relate to the development of secretory granules in these cells. The embryogenesis of the gastrointestinal endocrine cells, which form part of the DNE system, may be explored more readily with the aid of this marker. Third, the presence of this marker in the adrenal medulla of other animal species, such as monkeys and pigs, will enable investigators to develop and analyze animal models of diseases involving this neuroendocrine tissue.

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Nicarbazin Complex Yields Dinitrocarbanilide as Ultrafine Crystals with Improved Anticoccidial Activity

Abstract. *Nicarbazin, a drug used to control the protozoal disease coccidiosis in poultry, is a complex of the highly insoluble drug 4,4'-dinitrocarbanilide with 2-hydroxy-4,6-dimethylpyrimidine. The structures of this and other 4,4'-dinitrocarbanilide complexes have not been determined, but an analogous 2:1 complex of 4,4'-dinitrodiphenylamine with 1,4-diacetylpiperazine has been prepared in which the only possible bonds are hydrogen bonds between the amide carbonyls and amino hydrogens. Scanning electron microscopy revealed that micron-size crystals of nicarbazin disintegrate in water to form much smaller dinitrocarbanilide crystals. Similar complex dissolution in the gut of poultry may account for the greater effectiveness of dinitrocarbanilide when administered as complexed rather than uncomplexed drug. Particle size problems associated with other highly insoluble drugs and pesticides may be resolved by the use of nicarbazin-like complexes.*

In broiler production, prevention of infection by enteric protozoan parasites of the subclass Coccidia is of critical importance (*1*). Coccidiosis has effects on poultry ranging from reduced feed efficiency to high mortality, depending on the species of invading coccidia, strain pathogenicity, and other factors. The first agent found to give satisfactory broad-spectrum control was nicarbazin (*2, 3*), the 1:1 complex of 4,4'-dinitrocarbanilide (DNC) with 2-hydroxy-4,6-dimethylpyrimidine (HDP) (*4*). This is administered in the feed at a level of 0.0125 percent. Nicarbazin has been used as a coccidiostat for nearly three decades now. However, since the original reports on this drug, the phenomenon of potentiation by complexation which it illustrates has not been investigated to our knowledge.

On a weight basis, nicarbazin is at least ten times as potent as DNC in the control of *Eimeria tenella*, the main cecal pathogen of chicken coccidiosis. In trials with poultry maintained in batteries or pens, approximately the same ratio for potencies of complexed and uncomplexed DNC is observed for five species of *Eimeria* (*E. tenella, E. necatrix, E. brunetti, E. maxima*, and *E. acervulina*). Since HDP has no anticoccidial activity when used alone (*2*), its contribution as a complex component must be associated with improved absorption of DNC. A similar situation prevails with other complexes, such as DNC combined with 1,4-diacetylpiperazine (*5*), 3-methoxy-2(1H)-pyridinone (*6*), and 3-amino-1,2,4-triazine (*7*). The complexes are more effective than DNC and approach nicarbazin in potency, although the noncarbanilide components themselves have no anticoccidial activity.

Complexes of HDP with antibacterial and antifungal ureas, thioureas, and guanidines have been patented (*8*). These are claimed to be more potent than the uncomplexed biocides. For example, the toxicity of the *N,N'*-bis(3,4-dichlorophenyl)thiourea–HDP complex to *Staphylococcus pyogenes* in vitro is reported

to be 8 to 16 times that of the thiocarbanilide itself (*9*).

Nicarbazin is obtained by stirring, at room temperature, a suspension of bright yellow DNC in a methanol solution of a molar equivalent or excess of HDP. The reaction is complete within 30 minutes, as indicated by a change of the solid phase to bulkier, cream-colored crystals. Other complexes are made in the same way, although different solvents (such as acetone and ether) may be used and an excess of the soluble, noncarbanilide component generally is employed. Most complexes are 1:1 in composition, but 1,4-diacetylpiperazine combines with two molecules of DNC. The infrared spectra of these compounds are characterized by a sharp carbonyl band at 1735 cm^{-1}, in contrast to the overlapping bands of the original carbanilide at 1756 and 1735 cm^{-1}. All the DNC complexing agents cited are water-soluble. The complexes, although stable when dry, break down with regeneration of DNC when exposed to water, methanol, or other solvents. For this reason, recrystallization is impossible. Those complexes with good shelf life approach nicarbazin in their efficacy as coccidiostats.

In all probability, the complex components are linked by hydrogen bonds, but proof of this and identification of specific bonds have not been possible. Structure determination methods requiring study of the material in solution are unworkable because the complexes are insoluble in nonpolar solvents and decompose in bonding solvents with regeneration of DNC. Moreover, crystals suitable for x-ray diffraction cannot be obtained. Solid-state spectroscopic studies suggest that we are dealing with weak electron donor-acceptor complexes (*10*).

It seemed worthwhile to establish the minimum requirements for complex formation. To that end, a 2:1 complex of 4,4′-dinitrodiphenylamine (*11*) with 1,4-diacetylpiperazine was prepared. A suspension of 631 mg of 4,4′-dinitrodiphenylamine in a mixture of 3 ml of methanol and 3 ml of ether containing 468 mg of 1,4-diacetylpiperazine was stirred vigorously at room temperature for 4 hours. The orange-brown suspended material turned yellow. On filtration, 680 mg of product was obtained. Nuclear magnetic resonance and analytical data agree with formulation as a complex of two molecules of 4,4′-dinitrodiphenylamine with one molecule of 1,4-diacetylpiperazine. [Calculated percentages for $(C_{12}H_9N_3O_4)_2 \cdot C_8H_{14}N_2O_2$ are C, 55.81; H, 4.68; and N, 16.27; the percentages found are C, 55.88; H, 4.69; and N, 16.19.] This complex is analogous to nicarbazin and the $(DNC)_2$-diacetylpiperazine compound, but the bonding possibilities are more limited. All that are available for linkage are the components of two hydrogen bonds, the electron-rich amide carbonyls of the diacetylpiperazine moiety, and the electron-poor amino hydrogens of the dinitrodiphenylamine units. Their donor and acceptor properties must be sufficient for complexing. Our conception of the formation of this complex is diagramed in Fig. 1.

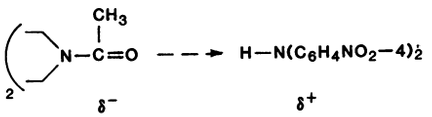

Fig. 1. Formation of 4,4′-dinitrodiphenylamine–1,4-diacetylpiperazine complex.

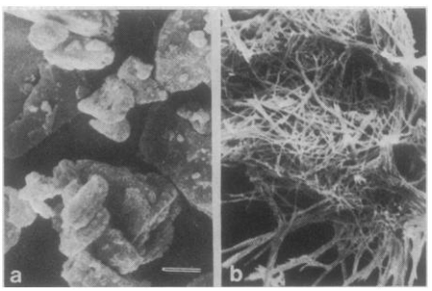

Fig. 2. Scanning electron micrographs showing (a) dry, micronized nicarbazin and (b) product resulting from immersion of nicarbazin in water for 5 hours (both ×5000; scale bar, 1 μm).

In addressing the biopharmaceutical role of nicarbazin and congeners, we will focus on the concentration of DNC in blood plasma. The relevance of this criterion is indicated by the fact that effective doses of DNC and nicarbazin produce similar concentrations in plasma (*3*, *12*). Moreover, Waletzky and Probst (*13*), by studying birds with ligated ceca, showed that nicarbazin can control an *E. tenella* infection via the circulatory system.

The superior activity of complexed DNC has been assumed to be due to its absorption as intact complex, but the facts do not support this view. One, two, and four hours after oral administration to chickens of nicarbazin (250 mg/kg), the molar ratio of HDP to DNC in plasma is 20 or greater (*12*), so only 5 percent of intact complex could have been absorbed. Indeed, a ratio of 20 is probably low for several reasons. First, the figure is based on the assumption that DNC is absorbed only as complex. Second, HDP actually is cleared more rapidly, according to other evidence in the study cited. Finally, the dose used is massive, about 20 times the usual daily level, while greater decomposition of complex in the gut is likely at a higher dilution. It seems reasonable to conclude that, under normal conditions, essentially complete decomposition of nicarbazin to DNC and HDP precedes absorption.

A crucial aspect of DNC absorption is the compound's extremely low solubility in water, only 2 μg per 100 ml. With a water-feed ratio of 2, birds receiving 100 ppm nicarbazin in feed have 2.3 mg of potentially available DNC present in 100 ml of gut contents, but only about one-thousandth of this would be in solution and available for absorption. According to Porter and Gilfillan (*12*), birds dosed for 12 weeks have a DNC content in plasma of 160 μg per 100 ml. Most of this must be bound to plasma protein. The DNC content drops to 63 μg 24 hours after drug withdrawal. It follows that approximately 100 μg per 100 ml has to be replaced daily to maintain the level in plasma. Clearly, the surface area of the solid DNC in the intestine must be a key factor in ensuring the necessary rapid turnover of dissolved drug. An alternative mechanism, the direct absorption of particulate DNC, has little or no precedent (*14*).

The low solubility of DNC indicates strong intermolecular bonding. Furthermore, as may be anticipated from such bonding, DNC crystals tend to aggregate. An earlier, suggestive observation was that submicron crystals are obtained by hydrolysis of DNC salts (*15*).

In nicarbazin formation, bonding between DNC and HDP replaces DNC intermolecular bonding. We propose that, when particulate nicarbazin is decomposed by wetting in the gut, the leaching of HDP leaves DNC molecules so separated that renewed intermolecular bonding results in formation of finer particles. Because of the increased surface area of the DNC so produced, its solution and absorption will be improved. Assuming a squared diameter–surface area correspondence, a three- or fourfold difference in particle size would account for the tenfold superiority of nicarbazin-generated over directly administered DNC.

Scanning electron microscopy (SEM) showed that nicarbazin does disintegrate in water to produce smaller crystals of DNC (Fig. 2). Assuming that ingested nicarbazin behaves similarly, this may explain the improved absorption of DNC administered as complex. A sample of

micronized nicarbazin was prepared for SEM by adhering the specimen to an aluminum stub, which was then carbon-coated and gold-coated. An identical sample of nicarbazin was immersed in water for 5 hours, vortexed, filtered, air-dried, and prepared for SEM in the same manner as the unwetted material. The water-treated nicarbazin (Fig. 2b) had much finer crystals of DNC than the dry nicarbazin (Fig. 2a). In a test run, DNC was observed to be unaffected by water immersion; the DNC used was factory-processed material that is wet-milled in methanol before being used in complex formation. The median diameters of dry and wetted DNC crystals were 0.51 and 0.53 μm, respectively, indicating no significant change, whereas the median diameter of DNC crystals produced from nicarbazin after immersion was 0.11 μm. In the size determinations, observations were made of 25 particles from each sample. Measurements were taken in a transverse plane at the anterior and posterior extremities, and diameters were calculated with the Zeiss MOP Videoplan. The four- to fivefold reduction in particle diameter, comparing factory-processed with nicarbazin-derived DNC, is not far from the reduction estimated as necessary to explain tenfold complex superiority.

Earlier recognition of a role for complexes in improved dissolution is evident in Higuchi and Ikeda's (*16*) study of a digoxin-hydroquinone molecular compound. However, since complexation and solution are related phenomena, recognition of such a role is also implied by biopharmaceutical interest in obtaining better absorption of material when solutions of poorly soluble drugs are administered (*17*). Generally, the emphasis has been on consistent performance with formulations of drugs soluble in the 1 mg per 100 ml range, rather than on enhanced activity. In no case has an improvement in efficacy been reported that is comparable to that found with DNC complexes, but here drug solubility is 1000 times less.

Our DNC observations suggest that the disintegration in water of crystals of hydrogen-bonded complexes formed from water-insoluble and water-soluble compounds will yield more finely divided crystals of the water-insoluble component. The same situation probably prevails on wetting of easily hydrolyzable salts of water-insoluble compounds. We suspect that another consequence of the replacement of the strong intermolecular bonding in highly insoluble compounds by complex or salt bonding is greater effectiveness in grinding operations. Other "particle size problems," as difficulties relating to the utilization of insoluble materials are often described, may yield to an approach involving the formation and dissolution of complexes.

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A Morphogenetically Competent Soybean Suspension Culture

Abstract. *A morphogenetically competent suspension culture was derived from embryonic axes of* Glycine max *cv. Mitchell. The cultural history included visual selection for nonfriable, embryo-like structures, recurrent selection in a regime of 2,4-dichlorophenoxyacetic acid exposure and withdrawal, and the replacement of the nitrogen in a Murashige and Skoog salts–based medium with 20 millimolar ammonium citrate. The embryoids produced by this suspension are capable of completing plantlet development. The suspension can be maintained by serial subculture.*

There are two routes of plant production from cell cultures: somatic embryogenesis and shoot organogenesis. For any particular culture, the choice of route is generally made on an empirical, operational basis (*1*) and, in most cases, if regeneration can occur at all, it occurs in response to only one of the two kinds of manipulations. Removal or reduction of high levels of auxin, usually 2,4-dichlorophenoxyacetic acid (2,4-D), leads to the formation of embryoids from certain cultures of certain species, while in other species exposure to a defined balance of auxin and cytokinin leads to the formation of shoots. Such manipulations, however, do not lead to plantlet production from cell lines of many species. We believe the physiological or epigenetic state of those cell lines is such that the cells are not competent for the induction of the processes underlying meristem organization. The molecular nature of the phenomenon, morphogenetic competence, remains obscure (*1*).

Wernicke *et al.* (*2*) maintain that in sorghum, and perhaps in other species, embryogenic cultures proliferate as small masses of suppressed primordia. They and others feel that high levels of 2,4-D suppress differentiation but allow continuance of a morphogenetically competent or determined state. For example, embryogenic suspension cultures of *Schizachyrium* require levels of 2,4-D in excess of the level giving optimal growth rates (minimal doubling times); lesser levels result in spontaneous plantlet formation in the suspensions (*3*).

Despite recent successes in achieving shoot or plantlet formation from explants, callus, or suspension cultures of various legumes, published reports on the genus *Glycine* are limited to shoot production from hypocotyl slices, multiplication of cotyledonary buds, and an incomplete somatic embryogenesis (*4–6*). The embryogenesis from suspension culture proceeds as far as late torpedo stage (*6*). Histological examination showed, however, that the embryoids were aberrant and lacked a well-organized shoot apical meristem. Such structures are termed "neomorphs" by Krikorian and Kann (*7*). Additional rounds of 2,4-D induction and transfer to em-