endogenous catecholamines and H^3-norepinephrine concentration. Blood pressure was also measured in rats during three repeated injections of tyramine as above and tachyphylaxis was demonstrated. After the third injection of tyramine, there was little or no blood pressure elevation, yet considerable amounts of the catecholamine were present in the heart. These observations indicate that the reduced response to tyramine after its repeated administration is a result of the depletion of bound catecholamines easily available for release.

To examine further the nature of the different types of binding of catecholamines, the effect of tyramine on H^3-norepinephrine in the heart for short and long periods of time was compared. Rats received H^3-norepinephrine as above and 30 minutes or 48 hours later 10 mg of tyramine per kg was given, and the animals were killed 30 min after the tyramine administration. After 48 hr, the specific activity of the norepinephrine was significantly elevated in the animals treated with tyramine (Table 1). Thus, tyramine preferentially released unlabelled norepinephrine. On the other hand, tyramine given 30 min after H^3-norepinephrine released labelled and unlabelled norepinephrine to about the same extent (Table 1). These results provide additional evidence for the presence of more than one pool of stored norepinephrine. After 48 hr the H^3-norepinephrine remaining in the heart is predominantly confined to stores having a slow turnover rate. Since tyramine results in an increase in specific activity at this later time, the catecholamines released must be derived from the stores that are turning over more rapidly and have a smaller percentage of the remaining H^3-norepinephrine.

Laboratory of Clinical Science,
National Institute of Mental Health,
National Institutes of Health,
Bethesda, Md., U.S.A.

LINCOLN T. POTTER
JULIUS AXELROD
IRWIN J. KOPIN

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Consideration of chemical reaction mechanisms in relationship to the biological action of "dual antagonists"*

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THE term "dual antagonists" was introduced by the author and his co-workers several years ago[1, 2] to describe certain chemical compounds incorporating the biologically essential structural features of two different, but synergistic, inhibitors into a single molecule. Use of such compounds as chemotherapeutic agents may be considered as a new form of "combination chemotherapy", a potentially more effective one because it provides for synchronized action and, possibly, more selective localization of the synergistic drug-components.[2] Somewhat similar suggestions were made by Wooley[3], who showed that "aggregate analogues" of *p*-aminobenzoic acid and 1,2-dimethyl-4,5-diaminobenzene inhibited the growth of *Staphylococcus aureus* in an "irreversible" manner. This relatively simple concept has been proving fruitful as a working hypothesis in the design of effective chemotherapeutic agents; additional results obtained through its application, and the conclusions derived from these results, are discussed in the present paper.

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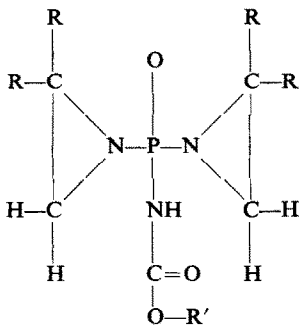
Several different types of compounds synthesized[1, 4] on the basis of the foregoing concept have shown significant inhibitory activities against various neoplasias in rodents[2, 5–7]. Two members of the N-[*bis*-(ethylenimido)-phosphoro]-carbamate series, AB-100 (I) and AB-103 (II), have undergone extensive clinical testing in various types of human malignancy and have caused beneficial results.[8–11]



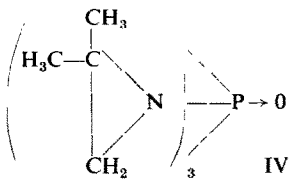
I. R = H; R′ = C_2H_5 ("AB-100")
II. R = H; R′ = CH_2—C_6H_5 ("AB-103")
III. R = CH_3; R′ = C_2H_5 ("AB-132")

In both of these compounds, however, the relatively high toxicity of the "alkylating" (ethylenimine) portion of the molecule has limited, to approximately 200 mg, the total dosage in which either of these drugs can be applied in man. It has been proposed that, at such a low dosage level, the "urethane' portion of the molecule could still contribute *via* synergism to the biological action of these drugs.[2] Furthermore, in the author's opinion, the pharmacological and clinical properties of these compounds tend to indicate that an important contribution of their carbamate portions may be that either of a "carrier" or a "localizer", or both, with desirable, and perhaps specific, membrane permeability properties. The differences in the human tumor "spectra" of these two compounds, which have identical alkylating groups but different carbamate portions, may be explained on this basis.

To test for a possible increase in the synergistic contribution of the urethane moiety to the antitumor action of an alkylating agent, the synthesis of a new series of ethylenimidophosphorocarbamates has been undertaken.[12] In these, the activity of the alkylating groups has been diminished deliberately by substitution at one or both of the carbon atoms of the ethylenimine ring with either alkyl or phenyl groups, or both. Such "bulky" substituents usually increase the stability of small ring compounds by steric hindrance. Presumably because of their diminished reactivity, these new "dual antagonists" are less toxic and can be administered at higher total dosage levels; thus, effective concentrations of the urethane moiety may be achieved.

Thus far, Ambrus and his co-workers at the Roswell Park Memorial Institute have studied thoroughly only the first member of this new series, "AB-132", ethyl N-[*bis*-(2,2-dimethylethylenimido)phosphoro]-carbamate (III), both in experimental animals and in human cancer patients.[13–17] The results of these studies have revealed striking pharmacological differences between this compound and its unsubstituted analog (I).[18, 13] Quantitatively, III is much less toxic than I in all species, and 10–50 times higher dosage levels can be employed. Furthermore, the LD_{50} of III in rats is about the same as in mice, while I is about three times as toxic in rats as in mice, the latter being an apparent characteristic of many known alkylating agents. Qualitatively, the toxic manifestations of III are quite different from those of I in the experimental animals, as well as in man. In the case of I, bone marrow toxicity is the dosage-limiting factor which, in the acute toxicity test, is the direct cause of death of the animals,[2] while in the case of III, the animals die of other causes, including the effects of inhibition of cholinesterases. Toxicity involving the central nervous system, rather than bone marrow depression, appears to be the limiting factor for III in most of the clinical studies reported[15, 17] (except in lymphomas and leukemias[16]); in addition, some of the manifestations (e.g. olfactory perversion[13]) are suggestive of the pharmacological effects of urethane.

For further comparison, in order to study the effect of substitution in the ethylenimine ring *per se*, various other 2,2-dimethyl-substituted ethylenimine derivatives containing no carbamate group also have been prepared[19] (including the 2,2-dimethyl homolog of TEPA, *tris*[1-(2,2-dimethylaziridinyl)]-phosphine oxide*, IV), and currently are being tested in animals.



IV

An attempt has been made to correlate some of the biological differences between these compounds with differences in their chemical mechanisms of reaction. Their hydrolysis reactions were studied first. Fig. 1 shows the rates of decomposition of the ethylenimine rings in CO_2-free distilled water, at

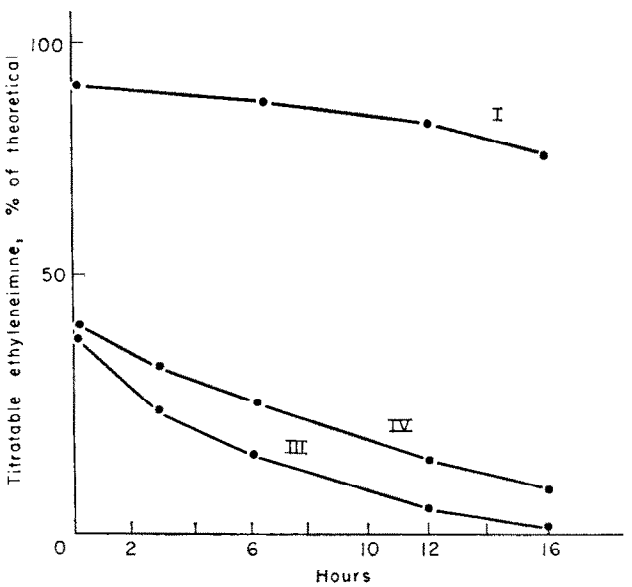


FIG. 1.

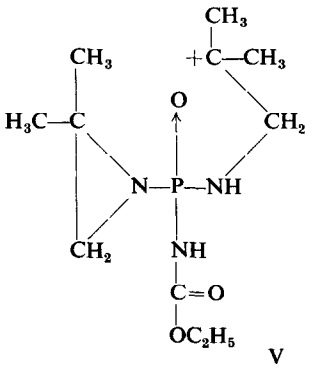
37 °C. Aliquots taken at various times were allowed to react with excess sodium thiosulfate, and the sodium hydroxide liberated in the reaction was titrated with hydrochloric acid. A similar method has been employed for the determination of epoxides and ethyleneimines.[19, 21] The reaction of Compound I with sodium thiosulfate proceeded to completion, (i.e. the calculated ethylenimine content remained), even after several hours incubation in distilled water, while less than one-half of the ethylenimine groups of compounds III and IV reacted at zero-time, and their reactivities rapidly decreased throughout the 16 hr of incubation. After 72 hr, the solutions were lyophilized and extracted with ether. Only III gave an ether-soluble fraction, and this was identified as chemically pure urethane.

The ether-insoluble hydrolysis products of all three compounds were purified by recrystallization. Structural studies[22] indicate that, in I and IV, the ethylenimine rings have opened to give the corresponding aminoalcohols still attached to the phosphorus through P—N bonds, and that the P—N bond linking urethane, in I, also remained intact. In contrast, III on hydrolysis not only "lost" its urethane group (see above), but also the P—N bonds linking the ethylenimine rings appear to have

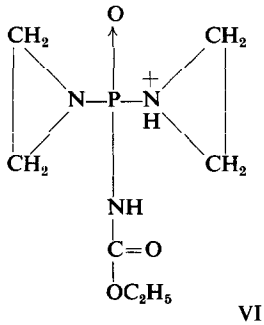
* Titrimetric assay of this compound has been reported,[20] but other information could not be found in the literature.

been broken, and the tertiary aminoalcohols formed from the latter re-attached to the phosphorus through new P—O—C and ionic (primary amine-phosphate) linkages. Final elucidation of the structure of this hydrolysis product is still in progress.

The unusual hydrolytic rearrangement of III can be explained on the basis of a carbonium ion (S_N1) mechanism, involving V as a reaction intermediate. In contrast, the hydrolysis of I appears to proceed by reaction of the relatively stable ethyleniminium ion, VI, which is similar to the reactive intermediates postulated for some other alkylating agents, including nitrogen mustard.[23]



V



VI

The different type of hydrolysis of IV, in comparison with III, indicates that not only the substitution at the ethylenimine carbons but also the carbamate portion of the molecule (absent in IV) has an effect on the chemical reactions of the alkylating function in this series of "dual antagonists". Moreover, substitution at the ethylenimine carbons (III) influences the hydrolytic liberation of urethane (in comparison with I). This chemical (kinetic) interaction of the two parts of the molecule is not identical, of course, with their "synergism" in the biochemical and pharmacological sense.

The author proposes (a) that differences in the chemical mechanism of alkylation may be achieved through substitution, at the carbon atoms of the ethylenimine rings, with either alkyl or phenyl groups, or both, and (b) that these differences are certain to manifest themselves in the relative rates of reaction with the various intracellular components; thus, important differences in the biological specificities of these compounds may be realized. Previous studies concerning the reaction mechanisms of alkylating agents in relationship to their biological activities have been published.[24]

As a result of these considerations, the author and his co-workers are continuing their studies concerning the reaction kinetics of variously substituted "dual antagonists" with model nucleophiles representing some of the important intracellular components. In these studies, attempts are made to determine, for each alkylation reaction, the relative contributions of S_N1, and S_N2 mechanisms, in an effort to correlate certain types of chemical reactions with the biological activity patterns of these compounds. Since 2,2-di-substituted (particularly, di-phenyl-substituted)-ethylenimine derivatives should be capable of forming relatively stable free radicals, the possibility of some small contribution of a third, i.e., homolytic alkylation, mechanism cannot be excluded; such a mechanism may play a major role if the formation of free radicals is induced by irradiation. For this reason, the effect of irradiation *in situ* upon the chemical reactions and biological activity patterns of these compounds will be explored.[25]

Department of Medicinal Chemistry,
School of Pharmacy,
University of Buffalo,
Buffalo 14, *New York.*

T. J. BARDOS

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