tagonist, 6-AN, to antagonize conversion of malate to citrate (Table II, Exp. 2). This level of 6-AN (2 mg/kg), by itself, has been shown to have no effect on the malate to citrate pathway(8). Previously, lethal levels of 6-AN were necessary to demonstrate a significant antagonism of the malate to citrate system in 755 tumor homogenates(8). Thus, stilbestrol, by reasons unknown, increases 6-AN toxicity to the 755 tumor without exerting the same degree of augmentation of toxicity to the host tissue. These preliminary enzymatic studies are correlated with the biological findings (Table I). How these findings are related to those of Ville *et al.*(9) and Hurlock and Talalay(10), who have previously shown a relationship between estrogens and pyridine nucleotide metabolism, is unknown.

Summary. 1) The data show that combination of hormone, stilbestrol, and anti-vit. 6-aminonicotinamide, has a greater anti-tumor effect than that produced by either drug alone. Further, addition of testosterone to the combination lessens host toxicity without altering the anti-tumor effect. 2) At an enzymatic level stilbestrol enhanced ability of this niacin antagonist to antagonize conversion of malate to citrate, suggesting a possible mechanism of action to explain the observed biological facts.

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Reversible Inhibition of 2,5-Alkyl Benzimidazoles on Chicken Sperm.* (25412)

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Alkyl benzimidazoles are known inhibitors of a variety of biological systems exhibiting a characteristic order of inhibition(1-6). Because of the nature of inhibition of virus multiplication and the characteristic inhibitory order, it has been suggested that the benzimidazoles inhibit a basic biosynthetic mechanism(s) involving nucleic acids and/or nucleoprotein(2,3,7). With these suggestions in mind, studies presented here were conducted to determine the effect of three 2,5-alkyl benzimidazoles on chicken sperm, a relatively simple biological system whose metabolic processes were assumed to involve mostly respiration.

Materials and methods. Pooled samples of semen were used. Semen was collected from Univ. of Maryland flightless strain males by massage technic. 2,5-Dimethylbenzimidazole (DMB) and 2-ethyl-5-methylbenzimidazole (EMB) were dissolved in 0.07 N HCl and 2-hepta-5-methylbenzimidazole (HMB) was

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dissolved in 0.118 N HCl at concentration of 10 mg/ml. Aliquots were added to 1/6 M phosphate buffer(8) to which semen was added at 1:9 dilution for motility determinations and 1:3 dilution for storage and fertility determinations. For fructose fermentation studies, as indicated by acid production, aliquots of inhibitor solutions were added to 1/3 M solution of fructose diluted with 1/6 M phosphate buffer giving final concentration of 2.25 mg/ml of fructose. Three parts of this solution were mixed with 1 part semen. These values were based on previous studies using pH depression as indicator of carbohydrate utilization(9). Acid production was determined, after incubation in stoppered tubes for 3 and 6 hours in 37°C water bath, by titration to pH 8 with 0.02 or 0.05 N NaOH. For fertility determinations, the semen-inhibitor-buffer solution was stored 3 days at 10°C. The inhibitor was essentially removed by diluting to 1:23 and 1:39 with buffer, centrifuged, and resuspended to original volume of 1 ml with Lake's solution(10). This sperm suspension was then inseminated into hens taking care not to exceed 1½ hours for washing and insemination process. The number of hens inseminated varied between 5 and 10/treatment. Controls were: a) fresh semen inseminated on day of collection to test fertilizing capacity of untreated semen, and b) stored semen diluted 1:3 with phosphate buffer minus inhibitors. Motility was determined 2 hours after semen was added to inhibitor-buffer solution and scored subjectively from 0 to 4 (0 = no motility; 4 = motility comparable to freshly collected semen). Removal of inhibitor in motility reversal studies was conducted in the same manner as in fertility studies except that sperm were held at room temperature 2 hours, diluted to 1:39 only, and resuspended to 1:9 dilution.

Results. Table I shows that the order of inhibition of chicken spermatozoa motility by benzimidazoles was DMB<EMB<HMB. The same inhibitory order was also found in fructolysis; DMB failed entirely to affect acid production and only doses of 300 μg or more of EMB were effective, while each dose of HMB (molar equivalents of 50, 100, and 200 μg EMB) progressively decreased acid production. It is evident that levels of DMB and EMB which completely inhibited motility had no effect on fructolysis. Motility was restored by simply washing the inhibited sperm with phosphate buffer. Restoration was complete for all cases except those levels which also inhibited fructose fermentation, *i.e.,* 300 and 500 μg EMB and each level of HMB (Table I). An additional washing of 300 μg EMB completely restored motility and increased that of 144 μg HMB from 2 to 3.

After 3 days storage of semen with doses of inhibitors, which partially inhibited motility but had no effect on acid production, fertility was greater than that of controls receiving no inhibitors (Table I). However, in cases in which inhibitor levels decreased acid production, fertility was decreased to 0. Thus, no fertility was obtained when HMB was added, since each dose decreased acid production. The relationship of fertility enhancement to motility inhibition was different for DMB and EMB; *i.e.,* fertility enhancement by DMB was directly related to motility inhibition, but for EMB these factors were inversely related. The increase in dilution from 1:23 to 1:39 of EMB-semen suspension with phosphate buffer for removing the inhibitor after storage appeared to increase fertility.

Discussion. The greater activity of EMB over DMB in inhibiting sperm motility and fructolysis is of the same inhibitory order as that found in other biological systems(1-6) thus adding further support to the suggestion that these compounds inhibit some basic metabolic mechanism(3). However, the marked increased inhibition of HMB over EMB suggests a difference in inhibitory action of 2-alkyl benzimidazoles (having alkyl chains longer than 2 carbons) on unicellular organisms as compared to multicellular ones since lengthening the chain past 2 carbons increased inhibition in bacteria(1) but failed to do so in chick embryos(4) or in virus multiplication on chick chorioallantoic membrane(2). This difference may be related to increased solubility in lipids with increased chain length, cell surface, and cell penetration.

The inhibitory action on motility of each level of inhibitors could be completely or partially reversed, depending upon inhibitor level,

TABLE I. Effect of Three 2,5-Alkylbenzimidazoles on Motility, Fructolysis, and Fertility of Chicken Sperm.

Inhibitor	μg/ml (total sol.)	Motility after 2 hr		Fructolysis* (ml)		Fertility after 3 days storage 1:23†		1:39†	
		Unwashed	Washed	After 6 hr, .05 N NaOH	After 3 hr, .02 N NaOH	% fert.	No. eggs	% fert.	No. eggs
Fresh semen						93	30	86 (80-90)‡	72
DMB	0	4	4	3.50	3.80			12 (5-19)	43
	100	3+	4	3.55	4.03			24 (18-30)	55
	200	3	4	3.51	3.93			26 (13-39)	51
	300	2	4	3.53	4.19			57 (40-74)	43
	400	1	4	3.43	4.42			60 (54-66)	48
	500	0	4		4.35				
EMB	0	4	4	4.35	4.00	8 (3-13)‡	45	5	20
	50	3	4	4.54	4.08	27	22	48 (43-52)	44
	100	2+	4	4.48	4.16	29 (13-44)	41	25	12
	200	0	4	4.39	4.79	0	48	5	19
	250	0	4	4.33		0	18		
	300	0	3	4.02	3.12	0	25		
	500	0	2	3.41	3.40				
HMB	0	4	4	4.42	3.90				
	72	0	3		3.41			0	29
	144	0	2	3.91	2.82			0	25
	288	0	2	3.28	2.12			0	27

* Acid production values corrected by subtracting quantity of acid added with inhibitors; 1 ml semen was used in 6 hr incubation and 0.5 ml used in 3 hr.

† Total dilution of semen after storage to remove inhibitors.

‡ Range of 2 exp. in parentheses.

by simply diluting out the inhibitor with buffer. The fact that fertilizing capacity was maintained after 3 days storage indicates that sperm life is prolonged by these inhibitors and that after removal they have no obvious, adverse after-effects. The highest average fertility results (48-60%) after 3 days storage are considered quite good for laying hens nearing completion of first year of production. Successful storage of chicken semen for 3 days has not previously been reported. Therefore, use of benzimidazoles or perhaps other inhibitors may permit successful extension of storage period beyond 3 days.

The ease with which these inhibitors are removed by dilution and the apparent freedom from after-effects has previously been reported on experiments in virus interference (7) and inactivation of newt larvae(6).

The fact that motility is completely inhibited by levels of DMB and EMB which have no effect on fructolysis indicates that these compounds are able to inhibit some metabolic process or mechanism which is intimately concerned with protein contraction and is independent of fructolysis. Prolongation of fertilizing ability is also independent of fructolysis. This conclusion is substantiated by the actual determination of fructose disappearance (Harris, unpublished) which showed that inhibitory levels of DMB and EMB which allowed maximum fertility had no effect on fructose utilization. Indeed normal fructolysis appears to be necessary during storage for maintenance of generative powers since levels which decreased acid production completely inhibited fertilizing capacity.

The exact process(es) interrupted by the

benzimidazoles is unknown but is undoubtedly complex. It has been suggested that they interrupt some biosynthetic mechanism involving nucleic acid and/or nucleoprotein(2, 3,7). The particular metabolites which are capable of partially reversing inhibition of certain benzimidazoles in various biological systems support this view: Vit. B_{12} is active in chick embryos(4), in *E. histolytica*(11), and in *E. gracilis*(12); adenine and guanine in other microorganisms(13); flavinadenine-dinucleotide and riboflavin phosphate in azo dye destruction by liver homogenates(5); and glycine in delta-amino-levulinic acid synthesis(14). If these compounds interrupt some biosynthetic mechanism at levels which prolong fertilizing ability and inhibit sperm motility, it would seem to follow that motility may be dependent upon this same aspect of biosynthesis and that its inhibition may prolong viability of sperm.

Summary. Motility, fructolysis, and fertilizing capacity of chicken sperm were determined following their subjection to various dose levels of 2,5-dimethyl-(DMB), 2-ethyl-5-methyl - (EMB), and 2 - hepta - 5 - methyl-(HMB) benzimidazoles. Motility after 1-2 hours and acid production from fructose after 3 and 6 hours were inhibited by the compounds with the following order of inhibition: DMB<EMB<HMB. Motility was completely inhibited by levels of DMB and EMB which had no effect on fructolysis. Fertilizing ability was prolonged by inhibitor levels which did not reduce fructolysis indicating that the disturbed metabolic process which inhibited motility was responsible for prolongation of fertilizing capacity and that this disturbance is not intimately concerned with fructolysis. The storage period for chicken semen was extended to 3 days with excellent fertility obtained with both DMB and EMB. The ease with which these compounds are removed by dilution and the lack of adverse after-effects appears to make them peculiarly useful for preservation of chicken sperm or possibly other types of cells or tissues.

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