must give erroneously high values. But on detailed computation this factor does not seem to account fully for the discrepancy between our mean values and those of Foldes and Murphy though it could account for some of the greater variability of the latter data.

It is well known that all or almost all of the cholesterol in erythrocytes is in the free form(1,2,8) whereas except in a few disease states the ester form is a constant fraction of about 71% of total cholesterol in plasma or serum. There is a rapid exchange of labelled cholesterol between cells and plasma(9,10) but apparently the cells offer only a fixed number of acceptor sites for free cholesterol and admit no ester cholesterol. Since when cells are laked the cholesterol in them all appears in the stroma(11), it follows that the cholesterol acceptor sites in the cells are in that portion. It seems reasonable to suggest that any inter-individual variability in cell cholesterol concentration must be related to individual differences in the relative volume or surface of the stroma per unit volume of the erythrocytes. All of the present data suggest that the stroma per unit volume of erythrocytes must normally be very constant although a very small real variability between individuals is indicated.

Summary. 1) Direct analyses of cholesterol in blood cells from 38 men yielded average value of 137.7 mg/100 ml and there was no correlation with serum values in these samples. Standard deviation of these cell values was only 6.5 and part of this small value is accounted for by analytical error. Indirect estimates agreed closely but variability was larger. 2) In 2 dietary experiments on 6 men which produced average changes of −63.8 and +51.8 mg of cholesterol/100 ml of serum, there were no significant changes in cholesterol concentration in the cells.

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Selective Inhibitory and Teratogenic Effects of 2,5-Alkylbenzimidazole Homologues on Chick Embryonic Development.*† (25842)

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Many studies have shown that compounds differing widely in molecular structure, as well as more closely related compounds, elicit specific teratogenic responses(1-7). In addition, compounds as closely related as homologues also have this ability as demonstrated by Ambellan(8) with phosphonucleotides in amphibians. Homologous alkyl benzimidazoles had a characteristic order of inhibition. Hendlin and Soars(9) first reported that increasing length of alkyl chain in the 2-position from 0 to 4 carbons caused a corresponding increase in inhibitory activity in bacteria. Similar results were found in chicken sperm with alkyl chains of 1 to 7 carbons(10). However, Tamm *et al.*(11) found that lengthening the chain from 2 to 4 carbons did not cause further increase in inhibition of virus multi-

plication; similarly, Blackwood and Shorb (12) found that the 7-carbon chain was no more lethal in the early embryo than the 2-carbon chain. Our purpose is to report selective teratogenic effects and inhibitory patterns of 2,5-dimethylbenzimidazole (DMB), 2-ethyl-5-methylbenzimidazole (EMB), and 2-hepta-5-methylbenzimidazole (HMB) when administered to chick embryos prior to incubation.

Materials and methods. The eggs were from 2 strains of White Leghorn hens (Mt. Hope and a random bred strain) mated to males of either Mt. Hope, random bred or flightless strains. Benzimidazoles previously mentioned were dissolved in dilute HCl and injected into the albumen through the air cell. Eggs were candled 5th day of incubation and daily thereafter to 18th day. Each infertile and dead embryonated egg was removed and immediately opened for examination. Dead or severely malformed living embryos were examined under stereoscopic microscope and staged according to Hamburger and Hamilton (13). All data represent arithmetic average of 2 to 5 repeats with approximately 15 fertile eggs/repeat. Control groups, which included non-treated eggs and eggs injected with dilute HCl (.06-0.1 N) or Vit. B_{12} dissolved in water, consistently displayed a low mortality, about 5% (average of 14 repeats, 286 eggs; no repeat exceeded 11%). Further details of storage and incubation, method of dissolving compounds, injection procedure and control groups have been previously described and discussed(12,14). Statistical analysis was not made because of the involved nature of the data. Since variation was inevitable, trends and correlation of different indicators of embryonic response are relied upon rather than absolute quantities. Embryonic response was determined by mortality, incidence of embryos ceasing development at successive periods throughout incubation, and types and incidence of types of malformations.

Results. A. *Inhibition patterns.* Plotting graphically the stages of development of dead or severely malformed early living embryos against cumulative per cent dead, curves of inhibition were obtained which followed a pattern characteristic of each compound. These graphs give 3 different types of information other than the general picture of the total curve: a) total mortality resulting from each treatment, b) incidence of embryos which ceased development at each stage period, and c) total per cent (thereby proportion) of embryos ceasing development at end of any period. Total mortality data were based on embryos dead through 18 days; however, development did not appear to proceed beyond approximately 14 days in those embryos dying between 14 and 18 days. When equivalent doses (0.50 mg DMB and EMB, 0.70 mg HMB which is approximately 3 μM) of the 3 compounds were thus compared (Fig. 1), EMB and HMB were similarly lethal but caused greater mortality than DMB. However, inhibition curves of EMB and HMB differed considerably while those of EMB and DMB were similar. For example, EMB caused a large proportion of embryos to cease development before stage 17 while HMB failed to stop development during this period.

Increasing doses of DMB did not increase mortality, and predominately inhibited development at 2 periods (stages up to 19 and 32-40) (Fig. 2). Contrary to this effect, dose increase of EMB (Fig. 3) caused a corre-

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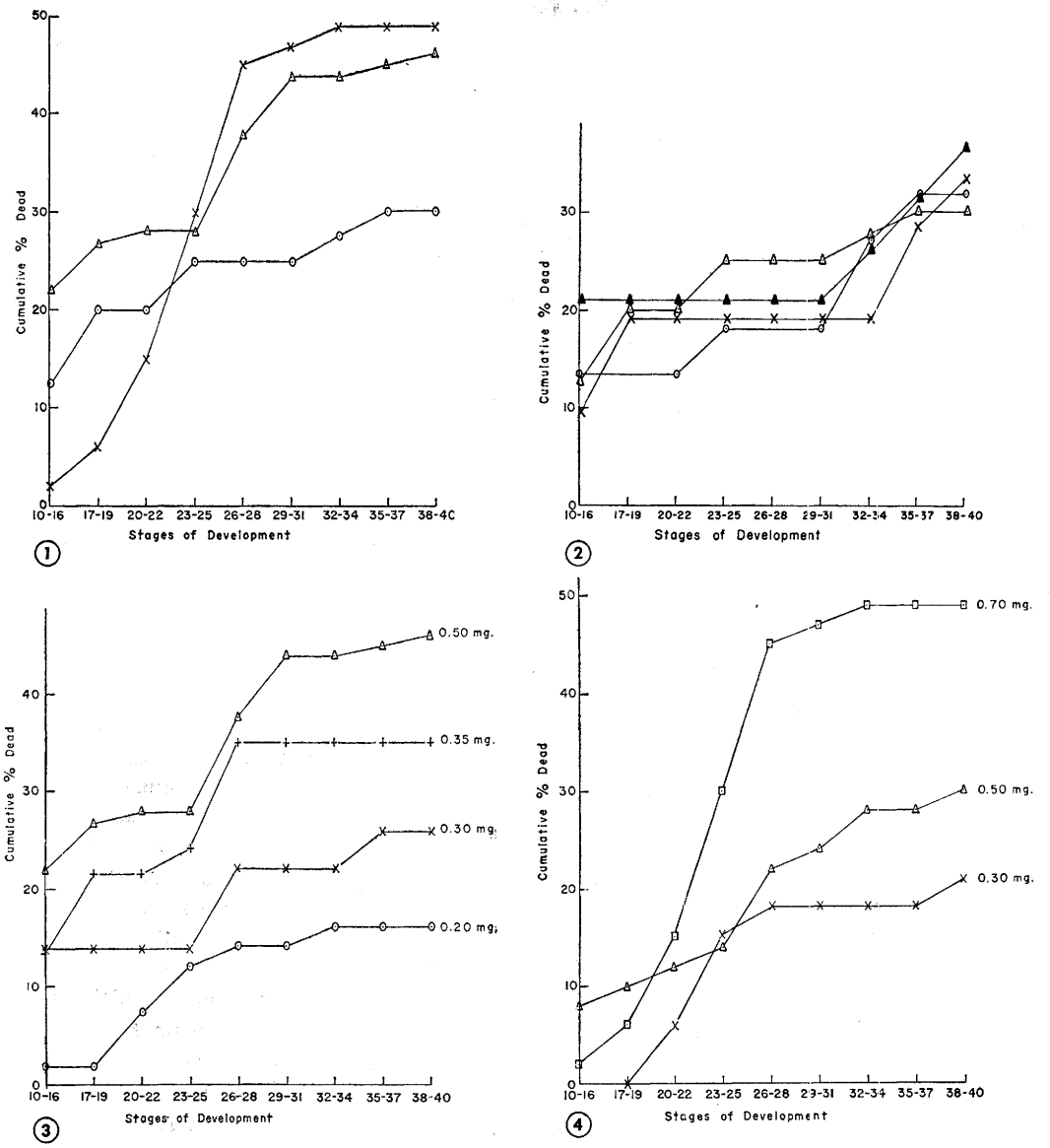

FIG. 1. Inhibition curves of DMB, EMB, and HMB when an approximately equivalent dose was administered to chick embryos prior to incubation. ×——× 3.0 μM HMB (avg of 3 repeats). ▲——▲ 3.1 μM EMB (avg of 5 repeats). ○——○ 3.4 μM DMB (avg of 4 repeats). Ordinate represents stages at which development ceased and abscissa represents cumulative % dead at times indicated. Stages are represented in groups of 3 from stages 17 to 40 (ca. 52-64 hr to 14 days). Stages 10-16 were combined because many embryos ceasing development during this period were too malformed to be adequately staged.

FIG. 2. Inhibition curves of several doses of DMB when administered to chick embryos prior to incubation. ○——○ 0.20 mg; ×——× 0.30 mg; ▲——▲ 0.40 mg; △——△ 0.50 mg. Each curve represents avg of 2-4 repeats. Explanation of graph is given under Fig. 1.

FIG. 3. Inhibition curves of several doses of EMB when administered to chick embryos prior to incubation. Each curve represents avg of 5 repeats. Explanation of graph is given under Fig. 1.

FIG. 4. Inhibition curves of several doses of HMB when administered to chick embryos prior to incubation. Each curve represents avg of 3 repeats. Explanation of graph is given under Fig. 1.

sponding increase in mortality and increase in percentage of embryos ceasing development in the early period. Also, these doses usually did not inhibit development appreciably after stage 28. Dose variation of HMB (Fig. 4) exhibited curve changes both peculiar to itself and similar to EMB. Increase in dose increased mortality and the highest and lowest dose predominately failed to inhibit development after stage 28. However, contrary to EMB, these doses failed also to inhibit development before stage 17. The middle dose inhibited development at almost every period through 40.

B. *Gross malformations.* These inhibitors produced numerous malformations. Table I summarizes malformations according to stage of the embryo at which development ceased. Since most types of malformations were associated with embryos of a limited embryonic age period, it is obvious that the incidence of the different kinds of malformations is reflected in the inhibition curves previously discussed.

Most dead embryos were malformed. However, several dead embryos resulting from highest dose of HMB and lower doses of EMB appeared grossly normal. Those embryos surviving treatments were not affected in any obvious manner. Chicks which hatched in several experiments were kept 2 to 3 weeks and were not different from the controls. Most embryos that ceased development at stages 10-16 and had a heart, were still living at 5-7 days as evidenced by pulsations of the heart. The area vasculosa (A.V.) of the anidian embryo (a well de eloped area vasculosa in the absence of an embryo), vesiculated embryos, and embryos in the form of an unidentified mass of tissue also were generally still living as indicated by redness of blood. Other malformations examined at this period were clearly defined and not degenerated.

Malformations of amnion and chorion were characterized by failure of the amnion to envelop the embryo in part or totally, and in some cases the amnion was completely formed but exceedingly small apparently causing an exaggerated ventral curvature of the embryo. In other cases the embryo was found within the yolk sac possessing no amnion. Many variations of torsion and flexion defects occurred. In some cases the embryo completely turned so that it lay on its right rather than left side or incompletely turned giving it an S-shape. In others, all or part of the embryo failed to turn but continued to lie on its ventral surface, or the head turned downward or upward in relation to yolk, or the embryo was in an E-shape. Flexion disturbances generally involved a failure of posterior trunk to curve ventrally in spite of the fact it lay on its left side. Many embryos killed by HMB exhibited the latter minor malformation. Disproportionate development of trunk and limbs often accompanied lordosis and involved ectro- or micromelia of all limb buds or one or both limb buds on one side, (left wing bud was most often affected), microsomia, or anouria or brachyouria. The "collapsed" optic lobe and eye were characterized by reduction in size of the structures and always occurred on right side and only in embryos of stages 26-31. This may have been a result of morbidity but did not occur in all embryos ceasing development at these stages. Exposed or extruded brain involved complete or partial absence of skull and a mushrooming effect of brain of stage 26-29 embryos. Beak malformations also varied considerably from slight twisting of beak to shortening of lower or an absence of upper beak. Beak malformations resulting from DMB were of the more severe type.

EMB produced the greatest and HMB the least number of different types of malformations (Table I). Malformations found in controls and produced by at least one dose of each inhibitor were: embryo formed as unidentified mass or vesicles (c), heart and trunk with greatly reduced or absent head region (g), affected torsion and flexion (n), microphthalmia (q), and beak malformations (v). Disturbance of torsion and flexion was the only malformation produced by every treatment. It is also evident (Table I) that certain malformations were produced by only DMB and EMB (*e.g.*, k and u) while other malformations were produced by only EMB and HMB (*e.g.*, f, l, and r), and several other

TABLE I. Incidence of Types of Malformations after Injection of Various Doses of DMB, EMB, and HMB into the Albumen Prior to Incubation.

Malformations occurring at different stages	Inhibitors: DMB Dose/egg (mg) .2-.3	EMB .2-.3	EMB .35	EMB .5	HMB .3	HMB .5	HMB .7	Controls
Stages 10-19	% of total malformations							
a. Anidian embryo	3.1	13.3	0	2.7	0	0	0	7.1
b. Small A.V. with embryo	0	6.7	2.7	8.1	0	0	0	0
c. Embryo as vesicles or mass	3.1	0	8.1	24.3	0	7.1	0	7.1
d. Heart—no or reduced head & trunk	0	0	5.4	8.1	0	0	0	0
e. Head—no or reduced heart & trunk	0	0	0	8.1	0	0	0	0
f. Heart + head—no or reduced trunk	0	0	5.4	5.4	0	14.3	0	0
g. Heart + trunk—no or reduced head	28.1	6.7	13.5	0	0	7.1	0	7.1
h. Vesicle in neural canal	0	0	5.4	0	0	0	0	0
Total	34.3	26.7	40.5	56.7		28.5		21.3
Stages 16-31								
i. Edema	0	6.7	2.7	2.7	0	0	0	7.1
j. Amnion-chorion malformation	0	0	2.7	5.4	0	0	0	0
k. Heart malformation	3.1	0	5.4	0	0	0	0	0
l. Visceral arch malformation	0	13.3	0	0	0	7.1	0	0
m. Open post. neural canal	0	0	0	5.4	0	0	0	0
n. Torsion & flexion defect	6.2	6.7	21.6	8.1	57.0	36.0	65.0	14.3
o. Lordosis	0	6.7	2.7	2.7	0	0	0	0
p. Dispropor. dev. of trunk & limbs	0	6.7	5.4	2.7	0	0	0	0
q. Microphthalmia	3.1	0	0	2.7	0	0	10.0	7.1
r. "Collapsed" optic lobe & eye	0	13.3	5.4	2.7	28.0	14.3	25.0	0
s. Other	0	0	0	0	0	0	0	28.6
Total	12.4	53.4	45.9	32.4	85.0	57.4	100	57.1
Stages 28-40								
t. Exposed or extruded brain	15.6	0	5.4	0	0	0	0	7.1
u. " viscera	6.2	13.3	2.7	0	0	0	0	0
v. Beak malformations	28.1	6.7	5.4	10.8	0	14.3	0	14.3
w. Other	3.1	0	0	0	14.0	0	0	0
Total	53.0	20.0	13.5	10.8	14.0	14.3		21.4
No. of fertile eggs	89	37	64	79	33	51	46	286
" " malformations	32	15	37	37	7	14	20	14
% malformations in fertile eggs	36	41	58	47	21	27	43	5

malformations were produced by only EMB (b, d, e, h, j, m, o, and p).

Discussion. This work demonstrates that very small changes in molecular structure of a chemical agent will elicit specific responses from the developing chick embryo, not only quantitatively but also qualitatively different. Mortality was least sensitive of the 3 criteria employed for determining embryonic response. By using mortality, inhibitory effectiveness could be obtained which differentiated between action of DMB and of EMB and HMB, but not between EMB and HMB. However, by determining inhibitory and teratogenic patterns, and in spite of the fact that EMB and HMB had equal lethal action, there was a great difference between inhibition patterns and types of malformations produced by these 2 compounds. Thus, the developing chick embryo responds in a specific manner to equivalent doses of each of the 3 alkyl benzimidazoles. This specificity is contrary to the implication that the action of these benzimidazoles differs only in degree of inhibition in that a characteristic order of inhibition has been found in a variety of biological systems (9,11,12,15,16). However, Tamm(17) found that DMB, EMB, and 2-butyl-5-methylbenzimidazole selectively inhibited to varying degrees, influenza virus multiplication as compared to toxicity to the chorio-allantoic membrane; and Blackwood and Harris(10) found a difference in motility inhibition of chicken sperm by EMB and DMB in relation to their ability to preserve generative powers; thus indicating differences in action other than degree of inhibition. Nevertheless, inhibitory and teratogenic patterns in the embryo indi-

cate that these compounds also have similarities as well as differences in action. One might conclude that these compounds interrupt multiple metabolic processes with some actions common to each compound while others are different. Reversal studies of these and other benzimidazoles indicate that their action(s) is indeed multiple(12,14,18-22).

It seems to be a characteristic of homologous series that members of the series produce distinct yet similar effects. This is seen here as well as in studies of Ambellan(8), who observed the action of mono-, di-, and triphosphorylated nucleosides on amphibian embryos. Other examples of this may be the paradoxical effects of homologues of trimethylalkylamines and several other compounds on neuro-muscular function and other physiological or metabolic processes. These effects of the latter compounds vary according to alkyl substitution; the shorter chains cause only muscle contraction, the longer chains only muscle relaxation, while intermediate chains cause both contraction and relaxation (23).

The numerous types of malformations and variable Vit. B_{12} reversal(12) obtained from the 3 benzimidazoles would seem to support the contentions of multiple action as well as interruption of basic process(es) in metabolism(15). It seems reasonable to assume that these two factors play very important roles in teratogenic action.

Summary. Inhibitory and teratogenic effects of various doses of benzimidazole homologues, 2,5-dimethyl- (DMB), 2-ethyl-5-methyl- (EMB), and 2-hepta-5-methyl- (HMB), were studied in the chick embryo following injection into the albumen prior to incubation. Three criteria of determining embryonic response were employed: lethality, inhibition curves derived from cumulative % embryos which ceased development at progressive stages, and types and incidence of types of malformations. EMB and HMB had about the same degree of lethality and were considerably more lethal than DMB. Inhibition curves from equivalent doses were different for each compound, and dose increase caused a different pattern of change in inhibition curves; thus, the embryo responds specifically to equivalent doses of each compound. Certain types of malformations resulted from all inhibitors, others resulted from only DMB and EMB, others from EMB and HMB, and still others from only EMB. The incidence of each different type of malformation was reflected in inhibition curves since most types of malformations were associated with embryos of a limited embryonic age period. Although there were differences in response to the compounds, there were also similarities.

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