## 3-Methylcholanthrene-Induced Embryonic Deformities in the Chick: Prevention by the Antioxidant, N,N'-diphenyl-*p*-phenylene diamine (DPPD)

It has been suggested that certain carcinogens, among them 3-methylcholanthrene (3-MCA), exert their carcinogenic actions by a free-radical mechanism[1-4]. Cancer occurs less frequently in mice which have high tissue levels of antioxidants or free-radical scavengers[1], and antioxidants, added to the diet of rats, prevent the appearance of cancers in animals fed the carcinogen paradimethylaminoazobenzene[3]. A principal reaction of the polycyclic hydrocarbons appears to be oxidation by hydrogen peroxide or by an oxygen atom yielding intermediary epoxides[5]. The 'carcinogenicity' of many aromatic hydrocarbons is believed related to a region of high electron density in the molecule[6,7], a condition which might easily occur in a free-radical[8]. Finally, there is a positive association between photodynamic toxicity of polycyclic compounds in *Paramecium caudatum* and carcinogenicity of the same compounds, an association which could be due to a free-radical interaction in each instance[9-12].

The chick embryo has suggested itself to us as a good model system in which to further test this hypothesis. 3-MCA implanted on the chorioallantois of developing chicks yields abnormal growth[13]. A lack of antioxidants in developing embryos (vitamin E deficiency) has an important detrimental effect on embryonic development; the introduction of antioxidants (including DPPD) prevents a number of congenital malformations expected in the vitamin E deficient animals[14].

Accordingly, the effects of 3-MCA on chick embryos, with and without pretreatment with DPPD, were investigated.

*Material.* Experimental procedures were carried out using White Leghorn chick embryos raised from fertilized eggs (DeKalb No. 151 Foundation stock of Truslow Farms, Chestertown, Maryland), maintained in an incubator at 60% humidity and 38 °C.

3-MCA was obtained from Distillation Products Industries of the Eastman Kodak Co., Rochester (New York). DPPD was supplied by the U.S. Rubber Company[15]. 3-MCA and DPPD were dissolved, for injection, in polyethylene glycol (PEG), U.S.P. 400, obtained from Fisher Scientific Products.

*Methods.* Injections of DPPD were made in the yolk 7 or 8 days after eggs were placed in the incubator; 3-MCA was injected into the yolk after 8 or $8\frac{1}{2}$ days of incubation. Injections were made with a $1\frac{1}{2}$ inch, 22 gauge needle, through a small hole cut in the shell over the air space. At comparable times, eggs were injected with PEG to test its effect. 4 groups were used: groups Ia and Ib (controls) were injected with 0.25 ml or 0.15 ml of PEG, respectively.

Groups IIa and IIb received DPPD (1.0 mg in 0.25 ml PEG and 1.0 mg in 0.15 ml PEG, respectively).

Groups IIIa and IIIb received 3-MCA; the amounts and dilutions were exactly as for DPPD in the groups above.

Groups IVa and IVb were injected with both compounds. A DPPD injection was followed by one of 3-MCA (separated by either 1 or $1\frac{1}{2}$ days). The concentrations injected were either 1.00 mg DPPD in 0.25 ml PEG followed by 1.00 mg 3-MCA in 0.25 ml PEG or 1.00 mg DPPD in 0.15 ml PEG followed by 1.00 mg 3-MCA in 0.15 ml PEG. The latter series (IVb) was run twice, on 2 separate occasions (IVb-1 and IVb-2).

The smaller injection volumes were used to reduce the total volume given when 2 injections were to be made. If 2 injections were to be made, embryos dying after the *first* injection were eliminated.

Following injection, eggs were returned to the incubator and candled daily. Dead embryos were removed, checked for beak and feet deformities, and fixed in 10% formalin. Following fixation they were staged according to Hamburger's stages and checked again for the same deformities. Only defects of the beaks and feet were scored; others may have been present, but, for the purpose of the present experiment, they were ignored.

Neither PEG nor DPPD produced any grossly observable abnormalities. PEG reduced the hatch to an average of 40–50% of expected, but all were alive on the 19th day of incubation. It is hatching that appears to have been affected. DPPD did not significantly further reduce the hatch.

*Results.* The results of 3-MCA treatment and of pretreatment with DPPD are given in the Table.

They were tested for significance by Fisher's Exact $2 \times 2$ Test[16]. The analyses showed that when the total number of affected embryos in group IIIa and IVa were compared, the reduction observed following pretreatment with DPPD is not significant. But the reduction in the number of beak deformities and deformities of one or both feet is significant at the 0.01% level. The comparison of the total number of deformities (not deformed embryos) between these groups is significant at the 0.001% level.

The differences observed between the repetitive runs of group IVb (IVb-1, IVb-2) were also tested and not found to be significant ($P = 0.87$ for total deformed and $P = 0.50$ for beaks and feet). These groups were then pooled and regarded as one. When compared to group IIIb, the reduction in the number of deformed embryos is again not significant, but the reduction in those of beak are significant at the 0.01% level; the reduction in those of 1 foot was not significant; the reduction in those of both feet was significant at the 0.005% level; and the


[1] D. HARMON, J. Geront. *16*, 247 (1961).
[2] D. HARMON, Radiat. Res. *16*, 753 (1962).
[3] N. M. EMANUEL and L. P. LIPCHINA, Acta Un. int. Cancr. *20*, 103 (1964).
[4] A. VITHAYATHIL, J. L. TERNBERG and B. COMMONER, Nature *207*, 1246 (1965).
[5] E. BOYLAND, Brit. med. Bull. *20*, 121 (1964).
[6] A. PULLMAN and B. PULLMAN, *La Cancerization par les Substances Chimiques et la Structure Moleculaire* (Masson, Paris 1955).
[7] B. PULLMAN, J. comp. physiol. Psychol. *64*, (Suppl. 1) 91 (1964).
[8] B. COMMONER, J. TOWNSEND and G. E. PAKE, Nature *174*, 689 (1954).
[9] S. S. EPSTEIN and M. BURROUGHS, Nature *193*, 337 (1962).
[10] S. S. EPSTEIN and M. SMALL, Cancer Res. *23*, 35 (1963).
[11] S. S. EPSTEIN, M. SMALL, H. L. FALK and N. MANTEL, Cancer Res. *24*, 855 (1964).
[12] S. S. EPSTEIN, I. BUBON, J. KAPLAN, M. SMALL and N. MANTEL, Nature *204*, 750 (1964).
[13] P. C. BEOHAR, P. GOLDHABER and A. T. HERTIG, Indian J. med. Res. *52*, 263 (1964).
[14] D. W. KING, J. Nutr. *83*, 123 (1964).


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[16] M. G. KENDALL and A. STUART, *The Advanced Theory of Statistics* (Hafner Publishing Co. New York 1961), vol. 2, p. 550.

| Experiment | | Day injected | No. injected | Embryos deformed No. % | Beak deformities No. % | Feet deformities one foot No. % | both feet No. % | Total No. of deformities |
|---|---|---|---|---|---|---|---|---|
| Ia | (1) 0.25 ml PEG | 8 | 8 | 0 | | | | |
| | (2) 0.25 ml PEG | 7 and 8 | 5 | 0 | | | | |
| Ib | 0.15 ml PEG | 7 and $8^1/_2$ | 22 | 0 | | | | |
| | Totals | | 35 | 0 | | | | |
| IIa | (1) 1.00 mg DPPD in 0.25 ml PEG | 7 | 4 | 0 | | | | |
| | (2) 1.00 mg DPPD in 0.25 ml PEG | 8 | 6 | 0 | | | | |
| IIb | 1.00 mg DPPD in 0.15 ml PEG | 7 ($8^1/_2$)[a] | 11 | 0 | | | | |
| | Totals | | 21 | 0 | | | | |
| IIIa | 1.00 mg MCA in 0.25 ml PEG | 8 | 7 | 7 (100) | 7 (100) | 2 (28.6) | 5 (71.4) | 19 |
| IIIb | 1.00 mg MCA in 0.15 ml PEG | (7) $8^1/_2$[b] | 16 | 16 (100) | 13 (81) | 4 (25) | 10 (62.5) | 37 |
| | Totals | | 23 | 23 (100) | 20 (86.6) | 6 (26.1) | 15 (65.2) | 56 |
| IVa | 1.00 mg DPPD in 0.25 ml PEG<br>1.00 mg MCA in 0.25 ml PEG | 7<br>8 | 14 | 12 (85.7) | 7 (50.0) | 2 (14.3) | 3 (21.4) | 15 |
| IVb | (1) 1.00 mg DPPD in 0.15 ml PEG<br>1.00 mg MCA in 0.15 ml PEG | 7<br>$8^1/_2$ | 15 | 9 (60.0) | 7 (46.7) | 3 (20.0) | 3 (20.0) | 16 |
| | (2) 1.00 mg DPPD in 0.15 ml PEG<br>1.00 mg MCA in 0.15 ml PEG | 7<br>$8^1/_2$ | 15 | 11 (73.4) | 6 (40.0) | 4 (26.7) | 3 (20.0) | 16 |
| | Totals | | 44 | 32 (72.7) | 20 (45.5) | 9 (20.4) | 9 (20.4) | 47 |

Experiments IIIa–IIIb: average No. of deformities/embryo = 2.44. Experiments IVa and IVb: average No. of deformities/embryo = 1.07. [a] 0.15 ml PEG injected at $8^1/_2$ days. [b] 0.15 ml PEG injected at 7 days.

reduction in the total number of deformities was significant at the 0.001% level.

Thus, DPPD an antioxidant, appears capable of preventing at least some of the abnormalities which 3-MCA produces in the chick, an observation which indicates that 3-MCA-produced abnormalities have come about through a free-radical mechanism. DPPD, MCA and a mixture of DPPD and MCA were then dissolved in PEG and, in darkness, pure oxygen bubbled through each. DPPD, and DPPD and MCA combined soon developed a dark-brown color, but the combination attained a deeper, stronger color much more rapidly. This indicates that the DPPD was oxidized by oxygen and that, in the case of the combination, this may have occurred by catalyzed oxidation involving a radical transfer reaction. It implicates 3-MCA in the role of this catalyst.

The data support a possible free-radical mechanism of action of 3-MCA in the production of embryonic defects. Whether its carcinogenic properties and its teratogenic properties come about by the same mechanism is not known. But, if they do, a free-radical mechanism may be common to both[17].

*Résumé.* Les embryons de poulet traités au préalable par du N,N′-diméthyl-*p*-phénylène diamine sont immunisés contre les déformations que produit le 3-méthylcholranthrène. Il est possible que cette protection soit due à une action neutralisante des radicaux libres formés par le 3-méthylcholranthrène avec l'oxygène.

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