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The toxicology of artificial colouring materials

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Synopsis—The toxicology of artificial colouring materials is discussed in relation to the many practical problems that arise in safety evaluation. The importance is stressed of adequate knowledge of chemical composition and reliable standardization of the specification of material to be tested. General analysis of metabolic changes undergone by colourings in the intestine, liver and blood of experimental animals is followed by consideration of systemic toxicity and carcinogenicity. Special attention is given to the question of tumour induction by subcutaneous injection.

Other aspects, such as effects on reproduction and the foetus, and mutagenesis are touched upon. Finally the question of dose-effect relationship is briefly considered.

Introduction

The use in the title of the term "artificial" might suggest that there is something especially toxic about such materials in comparison with colourings of natural origin. The simple fact is, of course, that what we do know about the toxicology of colourings relates almost exclusively to those of synthetic origin; while those derived from natural sources, however *naturrein* they may be, represent practically unknown territory from the standpoint of the toxicologist. Thus, while it is the object of this paper to discuss the various ways in which artificial colourings and their breakdown products or metabolites may exercise deleterious influences on the body, it should be emphasized that such knowledge constitutes the essential basis for the assessment of hazard and reliable evaluation of safety-in-use. It is left to the reader to decide whether the public is better protected when such fundamental information is available than when the safety of the material is taken on trust because "nature made it."

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It is my intention as far as possible to avoid covering ground that was very adequately dealt with at the 1963 Symposium on "Toxicology of Cosmetic Materials," and in particular my own contribution on "The assessment of safety-in-use: just how much is contributed by feeding tests in animals?" (1). I am fortunate also in having Prof. Calnan discussing reactions to colouring materials (2) and need not therefore elaborate on this aspect of their toxicology.

This discussion will be principally concerned with food colourings, for that is the field in which there is most knowledge and experience. It does not follow automatically that acceptance for food use renders such colours safe for use in cosmetics. Some mention will be made of the additional tests involved to establish suitability for incorporation into formulations intended for external application.

Specifications and impurities

I need hardly stress the importance of knowing what we are testing when we undertake to study the toxicology of a material. While specifications are available for many synthetic colourings, particularly those used in food, they usually fall far short of what is desirable from the standpoint of the toxicologist. Not for him the pathetic charade of limits for lead, arsenic or copper, archaic devices intended to ensure good manufacturing practice, and now quite wrongly interpreted as safeguards against toxic hazard. How adequate is the emphasis on limits for total amines or total ether extractable materials? This is the age of chromatography, and there is no reason why it should not be applied to separate, identify and ultimately to standardize the by-products that are present, so that their role in the production of biological effects may be accurately assessed. The time has long since passed when toxicological evaluation could be carried out "blind." If we are to make full use of recent advances in biochemical pharmacology we need to know all we can about the chemical composition of the material under investigation. A striking instance of the discrepancies between apparently authentic specimens of Ponceau 3R, recently reported by Hansen, *et al* (3), underlines the need to characterize most fully any colouring undergoing safety evaluation.

Metabolic changes

In the intestinal tract

Colouring matters entering the gut are subjected to the action of acid, digestive enzymes and the gut flora. The degradation undergone as a

result of the reductive fission of azo linkages in water-soluble colourings has been studied by Radomski and Mellinger (4) who showed that amines so formed in the rat were absorbed, metabolized and excreted in the same manner as the identical amino compounds given in free form by stomach tube. The most characteristic compound split off in this way is sulphanilic acid, which is absorbed and emerges in the urine as the free acid and its N-acetyl derivative (5). By administering the colouring by the oral route in some rats and intraperitoneally in others, one can judge the extent to which intestinal degradation takes place. Thus when Tartrazine is given by mouth, sulphanilic acid appears in the urine but no free colouring is excreted in urine or faeces. When the compound is given parenterally the animal is dyed bright yellow and free colouring appears in the urine, but without sulphanilic acid (6).

Whatever the extent to which a colouring is absorbed from the intestine, a complicating factor is the proportion of biliary excretion, which provides a direct route from the liver back into the intestine. Daniel (7) and Ryan and Wright (8) have shown that some water-soluble azo colourings are excreted almost quantitatively in bile. In a study of the relation of protein binding to biliary excretion, Priestly and O'Reilly (9) concluded that preferential binding to liver proteins, as against plasma proteins, was the determining factor – at least in the case of the colours studied by them. Biliary excretion has the effect of recycling the intact colouring, or the products derived from it, through the intestine, a process termed "enterohepatic circulation."

The stability of halogenated derivatives of fluorescein, given by mouth to rats, has been studied by Webb *et al* (10). Of the di-, tri- and tetrahalogenated colours, only the 4-iodo and 4-bromo derivatives were dehalogenated to fluorescein. Recoveries of unchanged material in the faeces were almost quantitative with tetrahalogenated derivatives, but fluorescein and its dihalogenated derivatives were cleared more slowly than the others. Webb and Brouwer (11) found that increasing halogenation of fluorescein diverts excretion from the urine to bile and hence the faeces, but also increases the total excretion.

Elevation of serum protein-bound iodine in man has been quoted as evidence that erythrosine is deiodinated after ingestion (12). However, since no effort was made to distinguish between protein-bound erythrosine-iodine and protein-bound iodine, this conclusion is unconvincing.

Sulphonated colourings that are not subject to attack by the

intestinal micro-organisms are sufficiently strong acids not to undergo appreciable absorption from the intestine. Thus the triarylmethane colourings are excreted almost quantitatively in the faeces (13). Nevertheless, some absorption does take place. Hess and Fitzhugh (13) found that less than 5% of the dose of colouring administered by mouth appeared in rat, rabbit or dog bile, probably reflecting the excretory pathway of absorbed colouring. Although it is stated that none of the colours were found in the urine, one wonders whether re-examination of the question by up-to-date techniques might not reveal the presence of metabolites in urine.

In the liver

Lipid-soluble azo compounds do not undergo cleavage in the intestine but are absorbed intact and acted upon by liver azo reductase to form the corresponding primary amines (14). Other changes undergone by such azo compounds involve protein-binding, hydroxylation and other effects such as N- and O- dealkylation brought about by microsomal processing (drug metabolizing) enzymes.

The subsequent fate of amines formed in the liver, or absorbed from the intestine, depends to a large extent on whether they are sulphonated and thus water-soluble, or whether they are alkyl-substituted anilines subject to extensive transformation within the body (15,16). In the former case they tend to be excreted intact in the urine or via the bile; or they may undergo minor conjugation such as acetylation before urinary excretion. The lipophilic amines are subjected to hydroxylation, or oxidation of alkyl substituents which probably proceeds through hydroxylation.

In the blood such acetylated amines or hydroxyamines exercise their well-known effect in inducing methaemoglobinaemia. This outcome has been recorded in man on numerous occasions following exposure to aniline or to "aniline dyes," as in children consuming coloured crayons. Methaemoglobinaemia is only the initial and, within limits, readily reversible change. Further transformation of haemoglobin leads to the development of erythrocytic inclusions termed Heinz bodies, with increased destruction of the affected red cells, the development of anaemia, repercussions on the bone-marrow and deposition of haemosiderin in the spleen and liver.

It is certainly true that in many instances these effects are only observed with high doses of colouring. Before one seeks to reach a

decision on what constitutes a maximum no-effect level, however, it is necessary to consider the following points:

(a) The sensitivity of the method used to measure methaemoglobin (bearing in mind the fact that the rat normally has about 1 g methaemoglobin/100 ml blood).

(b) The species differences in "resistance" to methaemoglobinaemia, presumably a reflection of the activity of methaemoglobin reductase (17). In this respect the cat is among the most sensitive species, man is almost as sensitive and the rat is a highly resistant species. Human genetically-determined deficiency of red-cell glucose 6-phosphate dehydrogenase creates a particular susceptibility to haemolytic amines, nitro compounds and the like (18).

(3) Similar variations in degree of susceptibility to the formation of Heinz bodies.

In our studies on food colourings, care has been taken to use a sensitive method for measuring methaemoglobin. In short-term feeding experiments carried out on rats, decreasing dietary levels of some colourings have given rise to Heinz bodies after increasingly long latent periods. At still lower dietary levels, although no effect was apparent, the application of a provocative test developed in our laboratories (19) revealed that a latent tendency to Heinz-body formation was still present. This example illustrates the errors that might have arisen from a mechanical, run-of-the-mill approach to safety evaluation, having as its objective the establishment of lack of toxicity rather than the discovery of the full facts.

A comparison of the rat's susceptibility to the three isomeric dimethylanilines and to the trimethylanilines mesidine and pseudocumidine (derived from Ponceau 3R) has revealed the greater potency of the trimethylanilines in producing haemolytic anaemia, methaemoglobinaemia and Heinz bodies (20).

General systemic toxicity

Gastro-intestinal effects, and specifically catharsis, was an early concern and led to the prohibition of some colours for food use. On a chronic basis in animals, impaired weight gain and liver and kidney damage have often been the most striking effects brought about by high doses. Liver damage may be associated in long-term experiments with the production of liver tumours, which will be referred to below.

Observations that present problems in interpretation are, firstly, the finding of an increase in relative liver weight in the absence of histopathological evidence of liver damage, even at the highest dietary level tested;

and, secondly, the presence of pigment granules in reticuloendothelial cells, especially of the liver, and/or in epithelial cells of the proximal convoluted tubules of the kidney. The significance of liver enlargement under these circumstances has been discussed (21). Storage of pigment granules that are neither haemosiderin, lipofuscin nor melanin, but probably represent the colouring in its original or a modified form, may reasonably be interpreted as an effect of exposure to high doses. In such cases there is no reason to believe that under the conditions of intended use any storage of this sort is likely.

Evidence of the uptake of certain dyes (not food colourings) in lysosomes lends particular point to the occasional occurrence of increased amounts of lipofuscin, particularly in the liver cells, after long-term feeding experiments. In the past such accumulations of what was called "wear and tear pigment" were ignored. Nevertheless the possibility exists that they represent the remains of previously-existing autophagic vacuoles which had been engaged in the task of restoring the integrity of damaged parenchymal cell cytoplasm.

Be that as it may, it must be realized that present-day methods of assessing toxicity to the liver, by means of tests of liver function and observation of histopathological changes, leave something to be desired – especially when the problem is one of detecting low-grade chronic irreversible damage. Moreover, the laboratory animal exposed to such agents as colours is not simultaneously exposed to the multitude of environmental toxic hazards by which man is surrounded in everyday life, and which may in certain circumstances exercise a synergistic or even a potentiating effect on hepatotoxic action (22).

Tests involving topical application

The availability of excellent reviews (23,24), makes it unnecessary to deal here with questions of methodology. Mention should, however, be made of special problems, such as hair dyes, application about the eye, inclusion of colours in dental products, and tests on abraded skin.

The effects of colouring materials on skin and mucous membranes of experimental animals are at most a preliminary guide to further tests of the complete formulation in animals and man. Consequently it is not proposed to deal with the subject further here.

Carcinogenicity

Tumour production by azo compounds has been the subject of many reviews. Clayson (25) distinguishes dimethylaminoazobenzenes, other

aminoazo compounds, derivatives of phenylazo-2-naphthol and other azo compounds containing neither amino nor hydroxyl groups. Of primary interest here is the group of colourings, which includes such compounds as Carmoisine and Sunset Yellow FCF, which are derivatives of phenyl- or naphthylazo-2-naphthol. Despite a few suggestive earlier observations, the weight of evidence today leads to the conclusion that food colourings of this class are not carcinogens.

Liver carcinogenesis by fat-soluble colours, of which *p*-dimethylaminoazobenzene is the prototype, and by water-soluble colours such as Ponceau 3R (26,27) creates considerable problems. Closely-related materials, for instance Ponceau 2R, are suspect; even if liver tumours are reported to have been produced by analogues of this sort, one cannot be certain that adequate steps were taken to exclude traces of carcinogen (i.e. Ponceau 3R) that might have been present as impurities in the product tested.

More fundamental, and more controversial, is the question – are low levels of such hepatocarcinogens acceptable for external topical application? Local carcinogenesis elicited by application to skin or mucous membranes is, of course, of special interest. Fortunately the colouring materials with which we are concerned do not produce such effects; nor has carcinogenesis, or promoting action being attributed to any of the colourings employed in food or cosmetics. Bladder carcinogenesis has been studied (25) but has merely confirmed other observations, such as the carcinogenicity of Ponceau 3R and the non-carcinogenicity of Ponceau 2R, Blue VRS and Patent Blue V (28). Weighing up the evidence, it seems reasonable to accept the use of colourings that are hepatotoxic or hepatocarcinogenic in animals, provided that use is severely restricted to preparations for external application where ingestion will not occur.

Tests by subcutaneous injection

For some unaccountable reason, synthetic colouring materials have been extensively tested for carcinogenic potential by repeated injection under the skin of rats. Of those colourings that have been investigated in this way, a substantial proportion have produced malignant tumours, usually fibrosarcomas, at the site of injection. It is likely that many, if not most, of the colourings not yet tested in this way are capable of inducing subcutaneous sarcomas.

The extensive background to this problem has been reviewed in considerable detail by Grasso and Golberg (29). When the host of complicating

factors has been taken into account it becomes clear that the production of local sarcomas by injected colourings is the indirect result of the peculiar circumstances of the test procedure. The colourings themselves, by causing tissue damage, create local lesions which are not in any way connected with carcinogenicity (30). From certain types of lesion sarcomas evolve, but this is a secondary and non-specific outcome only indirectly related to the nature of the colouring injected. Thus the resulting sarcoma provides no evidence, for or against carcinogenicity of the test material.

An analogy might be useful. There is the striking instance of the relationship between the production of oxalate bladder stones and bladder tumours in the rat. Ethylene glycol and polyoxyethylene-8-stearate (given at absurdly high dietary levels) have been labelled as carcinogens because they give rise to oxalate calculi in the rat bladder, and because – as a result of the presence of the calculi – tumours develop. Hueper (31) has denied that this is the invariable sequence of events, but the pathogenesis of bladder tumours under these circumstances has been firmly established by Weil *et al* (32).

The parallel with subcutaneous sarcomas is clear. Because they are injected repeatedly at a high concentration (2–4%) at frequent intervals (once or twice weekly) and always into the same site, some colourings produce local lesions. Once such lesions are formed by long-continued repeated insults to the tissues, development of sarcoma is an almost inevitable outcome. Yet any change in the conditions of administration – the same dose at lower concentrations, less frequent intervals, or injection at a number of sites in rotation – which serves to eliminate or greatly modify the local tissue reaction has a corresponding effect on sarcoma production (22). With increasing understanding of the pathogenesis of this type of tumour there comes a realization that by choosing appropriate conditions of administration one can cause any substance to produce sarcomas or, conversely, not to produce sarcomas.

From this assertion two conclusions follow. First, that the precise yield of subcutaneous sarcomas in any rat experiment has no relevance as an indication of carcinogenic potency. In the rat, the incidence of spontaneous tumours of this sort, or of tumours induced by the injection of saline, is so low that it is unjustifiable to regard the production of 18% sarcomas by Indigotine as non-significant while attributing carcinogenicity to Brilliant Blue FCF because the yield of sarcomas was 89% [results quoted from Hansen *et al* (33)]. The other conclusion which is inescapable

is that there is no basis for the argument that, in choosing safe colourings, less hazard is likely to be associated with the use of colourings that do not produce sarcomas than with the use of those that do. Superficially reasonable though it may be, this attitude simply evades the issue and perpetuates current misconceptions. Undoubtedly some true carcinogens do give rise to local tumours on subcutaneous injection; but in such cases their carcinogenicity has invariably been established by other means.

Effects on reproduction, toxicity to the foetus and teratogenicity

Very little information is available in this area of toxicology, except for acid disazo dyes such as Trypan blue, Evans blue and various types of Niagara blue. An excellent review has been published (34). While this group of dyes finds no application in foodstuffs, and probably none in cosmetics, the possibility exists that action on foetal development is an attribute of some part of the molecule of the disazo dyes. Beaudoin and Pickering (35) suggested that 1-napththylamine sulphonated in two positions, preferably 3 and 6, is a basis for teratogenicity. Accordingly Christie (36) tested the effect of 1,7diamino-8-naphthol-3,6-disulphonic acid given by the subcutaneous route to rats on day 8 of pregnancy. The maternal deaths, resorptions and developmental retardations observed were attributed to renal toxicity of the compound in the mothers rather than to any direct effect on the embryo. The present position, therefore, appears to be that foetal toxicity and teratogenic activity are confined to a special class of dyes given by injection in a variety of species. The relevance of these results to cosmetics colourings is doubtful.

Mutagenesis

The observation by German workers that Erythrosine had a slight but nevertheless genuine mutagenic effect on *E. coli* was followed up by Lück *et al* (37) who tested a variety of xanthene and other colourings. Xanthene itself, eosine, eosine BNX and erythrosine showed a very slight but statistically significant mutagenic effect on the bacteria when tested at a concentration of 10^{-4}M, supposedly the same order of magnitude as that used in foodstuffs. Erythrosine at 1%, and Rhodamine B at a 0.5% concentration had distinct mutagenic effects.

Induction of mutation in yeast has been reported by Nagai (38), using basic triphenylmethane dyes (such as methyl violet and *p*-rosaniline) and xanthenes (Pyronines Y and B). Although concentrations as low as 1 ppm or less produced effects, it is questionable whether these results have any significance from the standpoint of mammalian toxicology.

Dose and effect

The use of colourings in food is regarded as self-limiting and, subject to good manufacturing practice, no attempt is usually made to prescribe upper limits for the levels of application in particular foods. This factor, taken together with the possible presence of colourings in many articles of diet, consumed by both the very young and the very old, makes the task of safety evaluation much more exacting than it need be with cosmetics colours, where maximum levels of use can be prescribed for various applications. Thus Davis and Fitzhugh (39) were able to establish 0.05% as a satisfactory no-effect level for Lithol red (D & C Red No. 10) in rats.

Accepting that in such circumstances a reasonable safety factor (i.e. ratio between the maximum tolerated dose producing no ill-effects in animals and the likely total daily intake in man) such as 100 can be agreed upon, the levels of testing in animals are readily arrived at. Provided the maximum no-effect level is thus established in long-term lifespan studies in the animals, what happens at higher levels is of less importance as long as carcinogenesis is not involved. The colouring matters testing programme initiated by the Toilet Goods Association U.S.A., has been based on this approach (40).

Finally, there exists the need to discriminate between colourings incorporated in preparations whose use involves the likelihood of ingestion, and those products intended solely for external application.

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Introduction by the lecturer

One of the weaknesses of this paper is that it makes very little reference to cosmetic colours as distinct from food colours, and there are good reasons for this: the lack of published work, of which many of you must be aware; also the lack of practical experience in our own institute on these D & C and Ext. D & C colours. However, the general features are very much the same, and if one surveys the present situation there are really two key questions that one has to ask. Firstly, are the existing colourings, and particularly food colourings, capable of acting as sensitizers, and there Professor Calnan (41) gave us an indication.

The question has also been raised whether colourings in cosmetics or in food are doing harm other than by causing sensitization, but of this I do not think that we have any evidence whatsoever. There are, however, differences in metabolic routes when materials of this sort are ingested or are introduced into the body by other means. For example, in the case of oral administration of tartrazine, sulphanic acid is excreted in the urine of man and the rat but no intact colouring. There is reduction of the azo-linkage in the intestinal tract by the bacterial flora, with decomposition of the molecule into two halves. When the material is given intraperitoneally there is free colouring in the urine and no sulphanilic acid, i.e. the tartrazine goes through the system intact. It is conceivable that if a colouring is absorbed from the skin, the

(41) Calnan, C D., *J. Soc. Cosmetic Chemists* **18** 3 (1967).

intervention of the bacterial flora will only affect it when it is excreted in the bile. The point emerges that there is so much splitting of these azo-linkages in the intestine that it would appear advisable to make special studies of the amine moieties that are formed. The same moiety is common to a number of colourings, and by undertaking a close study of these individual moieties, as regards their further metabolism and other effects in the body, one would be automatically clearing the way for a better understanding of many of the other colourings which have not been adequately studied hitherto.

With regard to the question of specifications, a description of a colouring which was in order ten years ago is not really satisfactory today, and one has a right as a toxicologist to insist on an accurate description of these materials by the latest available methods.

As regards toxicological standards I think that the time has passed when the objective of toxicological testing was to bring about a negative result which acted as an assurance that everything was all right. Much more should be expected of toxicological testing, viz. a set of positive findings with regard to the metabolic fate and the influence of the test material on the animal. The effects of exposure are not necessarily toxic effects, but they are always there, and they should be found, because it is only by having positive findings that one has an assurance of safety. Purely negative findings leave the possibility that something of importance has been missed. We are often in a position now to derive positive findings by special methods if we really take the trouble, and this raises the question of a programme such as that of the Toilet Goods Association (U.S.A.) where the clearance of a number of cosmetics colourings is involved. If one examines what it is they have set out to do, one finds that essentially they are trying to produce negative results. I do not blame them for this. At present this is a valid procedure, but it does raise the question whether this particular approach is really sufficient. Of course, as I have mentioned, where one is dealing with colourings which are only to be applied externally, and there is little or no chance of ingestion, one obviously does not require the same standard of toxicological evaluation, the same stringency of examination, that is needed for colourings intended for food; all the same I think it is important to have an approach which seeks to provide a clear idea of what is happening rather than one that produces results which are almost completely negative.

There is one point which I should like to stress again, viz. the distinction between primary and secondary effects in toxicology. I have illustrated these effects by reference to subcutaneous sarcomas produced by colourings and other materials. A great many valuable colourings have thus been labelled as carcinogenic, and we in this country suffer particularly from this outlook.

Another important point I made, but only very passingly, is the fact that toxicological examinations are carried out on normal animals but in the case of man there is the chemical environment to be taken into account. By chemical environment I refer to natural constituents of food, other food additives, the exposure to various substances in the atmosphere, in drinking water, in the home, the consumption of drugs or alcohol. All these exposures have effects, many of which the body probably overcomes by physiological adjustments. The recognition of these changes, and the ability to distinguish them from pathological toxic effects often requires much more knowledge than we possess at present.