Quantitative Aspects of the Reaction of Some Carcinogens with Nucleic Acids and the Possible Significance of Such Reactions in the Process of Carcinogenesis

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Summary

Evidence for the reaction of alkylating agents, nitrosamines, azo-dyes, lactones, and hydrocarbons with nucleic acids in vivo is reviewed. Emphasis is placed on the quantitative aspect of such reaction, and on this basis the possible significance of the reaction with nucleic acids for the process of carcinogenesis is discussed.

Introduction

Carcinogenesis is perhaps the most interesting and important aspect of cancer research, yet ideas on this subject are in many ways still at the stage at which ideas on mutation were 25 years ago. About that time geneticists were agreed that mutations must react with some constituent of the cell so as to produce an inherited change in cell type, but were not agreed as to the nature of this constituent. At present there seems to be no general agreement as to the site of action within the cell of the carcinogen. Theories have been proposed which implicate the essential reaction as being with proteins (36, 61), DNA of the nucleus (8), some extrachromosomal or plasmagene factor (20) or with some element present in the wall of cells (5). The advocates of the viral theory of carcinogenesis would probably accept that reaction at any of the above sites could result in initiation of, or allow the propagation of, a pre-existing tumor virus within the cell.

Without wishing to discount other ideas, I shall in this review concentrate on the reaction of a number of different classes of carcinogenic agents with nucleic acids. I should particularly like to emphasize, in those cases in which data are available, the actual level of reaction found at biologically significant doses in terms of the number of molecules of the carcinogen reacted per molecule of DNA, RNA or protein. In order to express the results of reaction with nucleic acids for the process of carcinogenesis is discussed.

Alkylating Agents

In a recent review of the alkylating agents as carcinogens (14) it was shown that the published evidence to substantiate the claim that these agents as a class are carcinogenic was not extensive. Furthermore, some of the evidence has been criticized (47) on the basis that the tumors produced, namely pulmonary adenomas in the mouse, were insufficiently well defined to establish unequivocally the carcinogenicity of the compound used. However, allowing for these criticisms, it seems beyond doubt that a number of alkylating agents have been shown to be carcinogenic (77, 79) and that difunctional agents have a somewhat greater carcinogenic potency than the corresponding monofunctional agents.

Studies of the alkylation of nucleic acids in vitro (9, 11) showed that in both RNA and DNA the most reactive position is at N-7 of guanine and that with difunctional agents di-(guanin-7-yl) derivatives are formed (Chart 1a). Evidence has been presented (13, 34) to indicate that these latter compounds result from cross-linking of the double helix of DNA (Chart 1b). It has further been proposed (11) that the cross-linking of DNA is responsible for the cytotoxic action of difunctional alkylating agents since it prevents the strand separation necessary for the functioning of DNA as template for its own replication.

To determine the significance of cross-linking for the in vivo function of DNA, studies were made of the inactivation of T2...
and T4 bacteriophage by mustard gas (12). A single-hit inactivation curve was obtained (Chart 2) from which the mean lethal dose was calculated as being about 2-3 cross-links/total phage genome of $3.6 \times 10^4$ nucleotides, i.e., $1.2 \times 10^7$ molecular weight. With the corresponding monofunctional sulfur mustard (2-chloroethyl 2-hydroxyethylsulfide) the inactivation curve was of the multi-hit type and a much higher extent of alkylation was required to inactivate the phage (Chart 2). Studies (11) using the Ehrlich ascites tumor in the mouse showed that at a dose of sulfur mustard which prevented transplantation of the tumor the DNA was alkylated to the extent of 1 cross-link/4 $\times 10^4$ nucleotides, i.e., per DNA molecule of $1.5 \times 10^6$ molecular weight. Again for the sensitive strain of Escherichia coli B6-1, which lacks the ability to remove alkylated guanine moieties from its DNA, the mean lethal dose of sulfur mustard was shown (49) to be of the order of 1 cross-link/10$^6$ nucleotides, i.e., about 5 cross-links/total cellular genome of molecular weight $2 \times 10^9$.

In recent experiments (P. Brookes, A. R. Crathorn, P. D. Lawley, and J. J. Roberts, unpublished work), lymphoma cells, 5178Y, have been treated in suspension with $0.1 \mu g/ml$ of $^{35}S$-labeled sulfur mustard (specific radioactivity, 1050 $\mu$C/mg), this being a dose which ultimately kills about 90% of the cells (19). It has been possible to show that the isolated DNA has reacted in the same way as does DNA treated in vitro and that as expected the di-(guanin-7-yl) derivative was present to the extent of 22% of the total alkylation reaction. At this level of toxicity to the cells the extent of alkylation corresponded to 1 cross-link/mole-cule of DNA of mass $5 \times 10^8$ daltons.

It should be emphasized that in all the experiments described above the level of reaction of the alkylating agent with the DNA is of the same order as found for the cell as a whole, on the basis of moles/unit weight, and that RNA and protein are reacting to about the same extent, as measured in this way. We have no evidence for significantly outstanding reactivity of any 1 cell constituent towards the mustards. This means for example, that in a lymphoma cell, following alkylation with a toxic dose of sulfur mustard which produces about 1 alkylated guanine in each segment of DNA of mass $1 \times 10^8$ daltons, 1 in every 100 RNA molecules (of molecular weight $2 \times 10^9$) will be alkylated and 1 in every 1000 protein molecules (of molecular weight $1 \times 10^9$). These figures, along with the known relative insensitivity of enzyme action to alkylation, and the chemical nature of the reaction of these agents with DNA, strongly suggest that reaction at this site is responsible for the biologic action of alkylating agents.

All the results quoted above are concerned with cytotoxic
action since this is much easier to assess quantitatively than carcinogenic action, but it seems reasonable to assume that the process involved in carcinogenesis must follow from similar extents of cell alkylation. As mentioned above, the studies of Walpole et al. (80) and of Van Duuren et al. (77) indicated that the presence of 2 or more reactive groups, while not essential for carcinogenic activity, did enhance this property in a series of alkylating agents. This led to the suggestion (14) that mutation resulting from a gross deletion of genetic material, rather than point mutation, was involved in the process of carcinogenesis by alkylating agents. In this connection it may be noted that the majority of mutations observable in high organisms have been ascribed to deletion of, or gross damage to, DNA in contrast to the point mutations observed in microorganisms; the latter may not be detectable in more complex species (see for example the recent discussion by Kondo (48) of this question).

For a difunctional agent to transform a normal cell into a tumor cell the cell must obviously survive, and from the data given above this would only be expected to occur at very low extents of reaction. The recently published finding (49) that certain strains of E. coli which are relatively resistant to the action of difunctional alkylating agents, are able to remove enzymically the cross-links preventing DNA replication, is clearly relevant to this problem. The yet unpublished observation (P. Brookes, A. R. Crathorn, P. D. Lawley, and J. J. Roberts, unpublished work) of a similar mechanism operating in lymphoma cells, opens up a new field for consideration when discussing carcinogenesis. In bacterial cells it has been shown (P. D. Lawley and P. Brookes, unpublished work) that removal of the cross-linked guanine moieties is accompanied by degradation of part of the cell DNA which is subsequently resynthesized. For this to occur would seem to imply that the DNA double-helix must be broken at the point of excision of the cross-link, but without necessarily any loss of genetic information. Whether this leads to breakage and reunion of the DNA, or whether the DNA is held in position by protein until repair has taken place, is not known, but if similar processes occur in mammalian cells it may increase the possibility of loss of genetic material. Much further work is obviously needed before anything other than vague theorizing is possible, but the role of repair mechanisms operating at the genetic level may well be important in carcinogenesis.

**Nitroso Compounds**

The initial report by Magee and Barnes (53) of the induction of tumors by dimethylnitrosamine led to an extensive study of this and similar nitrosamines and nitrosamides. The result was the discovery of many carcinogenic agents whose activity was
demonstrated in many organs and species of animals (1, 29, 31). Liver, kidney, and lung tumors are induced by DMN in the rat (1, 53) and liver and lung tumors in the mouse (73, 76) and hamster (75). Even in the guinea pig, previously regarded as remarkably resistant to hepatocarcinogenesis, tumors of the liver have been induced by diethylnitrosamine (2, 32). Druckrey and his colleagues (28) in a study of over 40 different nitrosamines showed that in rats tumors of the liver, esophagus, tongue, forestomach, bladder, kidney, and lung were produced in almost all surviving animals, the site of the tumor depending on the structure, administration route, and dosage of the compound used. Although most studies have involved multiple applications, some compounds have induced tumors following a single dose (71).

As a consequence of the extensive work of Magee and his colleagues, and of Druckrey and his collaborators, it seems established that the dialkylnitrosamines are metabolized in the body so as to generate an active alkylating agent, which may be a diazaalkane itself or an active alkene or alkylcarbenium ion derived from it by further decomposition (30, 38). The mechanism for such a metabolism may be as shown in Chart 3.

Using initially 14C-labeled (54) and later 3H-labeled dimethylnitrosamine (50) it was shown that in vivo methylation of DNA, RNA, and protein resulted following injection of this agent into animals. Methylation of protein was also demonstrated in liver slices treated with 14C-labeled dimethylnitrosamine (55). Analysis of the isolated cell constituents showed that the products of alkylation, namely methylhistidines from the proteins, and 7-methylguanine from the DNA and RNA, were those expected from alkylation by a typical alkylating agent such as methyl methanesulfonate.

That the induction of tumors is a direct consequence of cellular alkylation seems strongly indicated. For example Lee et al. (50) have established some degree of correlation between the extent of methylation of an organ and its susceptibility to tumor induction in both rat and mouse. Furthermore, the finding of Lee and Spencer (51), that in newborn rats injection of DMN resulted in very low methylation of liver but relatively high methylation of kidney, correlates well with the finding of Terracini and Magee (74) that a high incidence of renal tumors, but only a low incidence of liver tumors, is produced by DMN in newborn animals. The study of Magee and Lee (56) in which effects of n-butyl-14C-methylnitrosamine and tert-butyl-14C-methylnitrosamine were compared following injection of these compounds into rats, showed that whereas the former caused methylation of cell constituents, the tert-butyl derivative did not do so. This would be predicted if the scheme of metabolism shown in Chart 3 is correct, since the absence of a hydrogen atom attached to the α-carbon atom in the tert-butyl radical would prevent the first step in the dealkylation leading to the active methylating agent. This failure of tert-butyl alkylnitrosamines to alkylate correlates with their failure to induce liver necrosis (37) or to produce tumors (27).

To summarize, it seems well established that carcinogenesis by these agents results from cellular alkylation, but which site within the cell is most critical still remains unresolved.

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Although most of the analytic work reported by Magee is concerned with alkylation of RNA, he emphasizes that this is largely for reasons of technical convenience, and that DNA and protein are also alkylated to almost the same extent (18, 54, 55). By analogy with the alkylating agents, this general reaction with all cellular constituents would be expected.

The problem arises of why the alkylating agents are less potent carcinogens than the nitrosamines, if both classes of agent cause the same type of cellular alkylation. The answer may lie in the quantitative extent of alkylation plus the degree of organ specificity shown by the nitrosamines. It is not possible to calculate the extent of alkylation of nucleic acids from some of the earlier experiments reported by Magee and his coworkers, in terms of molecules of methylated base/molecule of nucleic acid, but it is obviously fairly extensive. Magee and Schoental (57) report that, at the time of maximum reaction following treatment by injection of a rat with a single dose of 30 mg/kg of dimethylnitrosamine, 1–2% of the liver RNA guanine moieties are methylated. This extent of reaction is clearly several orders of magnitude higher than that attained at the median lethal dose of a difunctional agent for mammalian cells and, even with the much less toxic monofunctional agents, it is doubtful if it would be possible to achieve anything approaching this extent of alkylation of the liver or kidney without killing the animal as a result of alkylation of the cellular constituents of the hematopoietic system. Very relevant to this point are some recent experiments of Swann, Bradford, and Magee (personal communication) in which a comparison has been made of the methylation of nucleic acids isolated from organs of rats following administration of dimethylsulfate 14C or N-nitroso-14C-methylurea. The dimethylsulfate was injected i.v. at a median lethal dose while the N-nitroso-methylurea was given both p.o. and i.v. at a carcinogenic dose. It was found that both agents caused the methylation of RNA and DNA, but that the extent of methylation achieved with the noncarcinogenic dimethylsulfate was very much lower than that obtained following treatment with the highly carcinogenic N-nitroso-methylurea. The latter compound gave the same order of nucleic acid alkylation as previously obtained in kidney and lung of rats given a single carcinogenic dose of DMN.

These authors believe that carcinogenesis requires a certain level of methylation to be achieved in any organ, and that this threshold is at about 0.1–0.2 mmole of methyl/nucleic acid molecule. This is the level of DNA and RNA methylation achieved in the kidneys of rats following a median lethal dose of DMN, and which results in the development of kidney tumors in about 20% of the survivors. The liver of these animals which is very heavily damaged but in which tumors do not develop yields nucleic acid which is methylated to about 10 times the level found for the kidney.

The fact that such high levels of alkylation of individual organs can be achieved with the nitrosamines is almost certainly due to the tissue localization of the enzymes involved in converting the relatively nontoxic dialkyl nitrosamines themselves into the highly toxic alkylating moieties. The rapid reaction of these latter entities results in their not entering the circulation and so prevents damage to sites remote from that in which they are generated.

Quantitative data on the extent of protein alkylation obtained on treatment of animals with nitrosamines is not available from...
the earlier papers of Magee and his coworkers since an unknown proportion of the radioactivity found in the proteins isolated resulted from utilization of formaldehyde formed by N-de-methylation of methylnitrosamines. However, Craddock (17) states that only 0.02% of the available thiol groups of liver proteins are alkylated by DMN at a median lethal dose and contrasts this with the much greater extent of protein alkylation caused by methylidode which has not been shown to be carcinogenic.

The wide range of nitrosamines showing carcinogenic action and the varying specificity of site at which they produce tumors suggest that within this series it may be possible ultimately to correlate the carcinogenic property with alkylation of either RNA, DNA, or protein and so move 1 step nearer to elucidating the chemical change involved in the transformation of a normal to a tumor cell.

Lactones

Interest in the lactones as carcinogens is fairly recent and started with the report by Walpole et al. (80) of the induction by β-propiolactone of sarcomas at the site of injection in rats. The same substance was later shown to cause induction of skin tumors in mice (69). Since that time Dickens and his coworkers have investigated a large series of 4-, 5-, and 6-membered lactones including some naturally occurring substances and found many to be carcinogenic (24). Dickens concludes that the carcinogenic compounds are those in which the chemical reactivity of the lactone group is increased either by its being in a strained ring system or by its being conjugated with a carbonyl group or double-bond system.

Studies pertinent to the reaction of lactones with macromolecules are limited. β-Propiolactone has been shown to react with cysteine (23, 25) yielding S-(2-carboxyethyl)cysteine, and with guanosine (67) giving, after hydrolysis, 7-(2-carboxyethyl)guanine. This latter alkylation is clearly analogous to the reaction of ethylene oxide and guanosine previously described by Brookes and Lawley (9) and illustrates that the strained ring lactones are reacting in this respect like the alkylating agents.

Evidence of in vivo reaction of β-propiolactone with DNA of ascites tumor cells was reported by Richardson and Boutwell (66) who found that hydrolysis of the nucleic acid gave a product chromatographic and fluorescent properties of which suggested it to be the 7-substituted guanine derivative. Recently it has been found (P. Brookes and P. D. Lawley, unpublished work) that application of 3H-labeled β-propiolactone to the backs of mice results in radioactive labeling of RNA, DNA, and protein isolated from the skin 1 hr, 4 hr, and 24 hr after treatment. The level of nucleic acid reaction found of approximately 9 μmoles of β-propiolactone/nucleic acid nucleotide (i.e., per phosphorus atom) is of the same order as that found for the binding of carcinogenic hydrocarbons following a similar application to mice (Table 1).

In summary it is likely that the carcinogenicity of the lactones results from chemical reaction with a cellular constituent, but much additional work is required to establish firmly the nature of such a reaction.

Azo-Dyes

The feeding over a prolonged period of 4-dimethylaminoazobenzene to rats leads to the induction in high yield of liver tumors, while similar treatment of mice gives only few tumors, and guinea pigs, chipmunks, squirrels, and rabbits yield no tumors (62). This specificity of site and variation between species makes this and similar compounds suitable for studies in experimental carcinogenesis.

The early work on the metabolism of the azo-dyes and the binding of such metabolites to the proteins of the liver has been reviewed by the Millers (61, 62). As a direct consequence of this work the protein deletion theory of carcinogenesis was proposed, and has become sufficiently well-known as to require little further comment.

One feature of the work of the McArdle school was the lack
of any evidence of binding of the dye to nucleic acids (61) and the same conclusion was reached by Berenbom (4). However, as stated by Roberts and Warwick (68), the earlier work was based on spectroscopic methods and so would not detect the binding of any colorless dye metabolites resulting from reduction of the azo-linkage, while the specific activity of the 14C-labeled dye used by Berenbom (4) would not allow the detection of low levels of binding.

In their reinvestigation of the problem Roberts and Warwick (68), using very high specific activity 3H-labeled DAB, present evidence for the covalent binding of dye metabolites to RNA, DNA, and protein of rat liver following a single i.p. injection of 150 mg/kg of dye. An extension of these studies to binding of dye in other organs and to transplanted hepatomas in the rat, and to liver and spleen of the guinea pig, suggested that although there was no correlation between protein binding and carcinogenesis, some evidence of such a correlation did exist for binding to RNA.

The quantitative data obtained by Roberts and Warwick show that at the time of maximum binding, following a single injection of dye, the extent of binding, expressed as m/nmols/gm dry weight of the DNA, RNA, and protein of rat liver, was: for DNA, 22; RNA, 170, and protein, 700. When these figures are expressed as molecules of dye bound to each molecule of DNA (taking a molecule of 20,000 nucleotides, molecular weight 6.7 \times 10^9), RNA (molecular weight 2 \times 10^9), and protein (molecular weight 100,000), they become: for DNA, 0.15; RNA, 0.34, and protein, 0.07 molecule dye/cellular macromolecule. The values found for DNA and RNA are obviously significant, and of the same order as found for binding of other carcinogens as discussed above.

Marroquin and Farber (59) also reported the binding of 14C-labeled DAB to rat liver RNA. The extent of binding found by these authors expressed again as mnmols/gm were for RNA, 11, and protein, 9, that is 0.01 and 0.0009 molecule of dye/cellular macromolecule, respectively. The corresponding values for the cotton rat, which is not susceptible to butter yellow carcinogenesis, were for RNA and protein, 0.003 and 0.001 molecule of dye/cellular macromolecule, respectively.

Marroquin and Farber (58) also examined the binding of the related 2-acetylanilinofluorene-9-14C to rat liver RNA and protein following injection of Wistar rats. From the figures presented by these authors the binding found can be calculated to be 0.05 and 0.003 molecule of carcinogen/molecule of RNA and protein, respectively.

While admitting certain attractive features of the protein deletion hypothesis, especially in view of the recent ideas on suppressor proteins (45) and allosteric mechanisms of cellular control (63), the results discussed above show that reaction of azo-dyes with nucleic acids does occur and may still prove of fundamental importance in the induction of tumors by these agents. This point is fully discussed by Roberts and Warwick (68).

**Hydrocarbons**

As early as 1938 Fieser (33) postulated that polycyclic hydrocarbons might be metabolized in vivo to derivatives which could bind to cellular constituents. The first evidence of a hydrocarbon protein binding was that of Doniach et al. (26) using 3,4-benzo-pyrene. The subsequent work of Miller (60) and of Heidelberger et al. (41, 81) confirmed the hydrocarbon-protein binding and seemed to extend the application of the protein deletion theory. These views of hydrocarbon carcinogenesis have been recently stated very clearly by Heidelberger (39).

There are, however, several observations which appear to be most easily explained on the basis of an interaction between hydrocarbons and the nucleic acids. The finding of De Maeyer and De Maeyer-Guignard that the synthesis of interferon (22) and the replication of DNA viruses (21) are inhibited by hydrocarbons suggested a similarity to the action of actinomycin which is known to inhibit RNA-dependent DNA synthesis through an interaction with the DNA. Jensen et al. (46) showed that, 7,12-dimethylbenz(a)anthracene selectively damaged mammalian cells that were undergoing active DNA synthesis and that incorporation of H-thymidine into DNA was depressed.

The observation that 3-methylcholanthrene or 3,4-benzyrene stimulated microsomal enzyme activity in rat tissues and that this effect was inhibited by actinomycin D led Loeb and Gelboin (52) to conclude that the hydrocarbons affect the gene-action system at the level of nuclear RNA synthesis. The significance of these findings to the problem of carcinogenesis was indicated by the report of Gelboin and Klein (35) that actinomycin D inhibited the carcinogenic action of hydrocarbons on mouse skin. Hsu et al. (43) while observing an inhibition by hydrocarbons of bacteriophage replication in E. coli spheroplasts, suggest that the mechanism of viral inhibition differs from that produced by actinomycin in that RNA virus as well as DNA virus production is inhibited and furthermore that host cell RNA synthesis is unaffected by the hydrocarbons.

Although these series of results are most easily explained in terms of direct reaction with DNA, it is appreciated that they could also be explained in terms of reaction with a repressor protein since such a reaction can be envisaged as resulting in either stimulation or depression of cell synthetic activity. However, it remains to be established that cellular control in mammalian systems results from the same mechanisms as found in bacteria.

Although in some early work Heidelberger and Davenport (40) reported the binding of 1:2,5:6-dibenzanthracene-9,10-14C to RNA and DNA of mouse skin following its application to the backs of mice, a subsequent reinvestigation by McKinney and Heidelberger [unpublished in detail but quoted by Heidelberger (39)] claimed that the earlier results were due to insufficient purification of the nucleic acids. However, following the report of Brookes and Lawley (15) of nucleic acid-hydrocarbon binding, this criticism was withdrawn and the DNA-hydrocarbon binding confirmed.

Brookes and Lawley (15) used a series of tritium-labeled hydrocarbons of varying carcinogenic activity and studied their binding to carefully purified DNA and RNA as well as to a crude protein extract of mouse skin, following a single application to the shaved backs of mice. As a final stage of purification the DNA was treated by ultracentrifugation in a gradient of aqueous cesium chloride while the RNA was fractionated by sucrose-gradient centrifugation (Chart 4). In both cases the O.D. and radioactivity curves were coincident and the nucleic acids were isolated from the gradients retained the bound hydrocarbon.

**Reaction of Some Carcinogens with Nucleic Acids**
The results of a large series of experiments showed that for each individual hydrocarbon there was a consistent pattern of binding to the RNA, DNA, and protein but variations were observed between different hydrocarbons. A study of these variations showed that, whereas the extent of binding of protein or RNA bore no correlation with the carcinogenic potency as expressed by Iball’s index (44), such a correlation did exist for DNA binding, whether expressed as the ratio of the specific radioactivity of DNA to that of RNA or protein, or as the number of molecules of hydrocarbon bound/molecule of DNA following a dose of 1 μmole/mouse.

Since the initial publication (15), 2 more hydrocarbons, namely, 1:2-benzanthracene and 1:2-benzopyrene, have been studied and shown to fit the observed relationship (Chart 5). Recent studies (P. Brookes and P. D. Lawley, unpublished work) have eliminated the possibility that the radioactivity associated with the nucleic acids is due to breakdown of the hydrocarbon and reutilization of the label for de novo nucleic acid synthesis. It was found that on degradation of DNA or RNA labeled with 7,12-dimethylbenz(a)anthracene-3H and subsequent chromatography on paper the radioactivity was not associated with the normal bases but was found mainly as a single spot which remained at the origin, in contrast to added unchanged hydrocarbon which ran at the solvent front in the system used.

In attempts to obtain a source of nucleic acid-hydrocarbon complex which was more convenient than mouse skin, Hela cells in tissue culture and ascites tumor cells in the mouse were treated with tritium-labeled hydrocarbons, and nucleic acids isolated. In each case a binding pattern similar to that observed on mouse skin was found, but the level of binding was of the same order as that found in skin.

In view of the availability of mutants of E. coli differing in their response to agents known to react with the cell DNA (42) it was clearly of interest to attempt to get hydrocarbon bound to the nucleic acids of this system. Initial experiments in which the hydrocarbon was added to the bacterial suspension in benzene solution or solubilized in either bovine serum albumin or tetramethyluric acid gave absolutely no binding to any cell constituent. However, in very recent experiments when the hydrocarbon was added in dimethylsulfoxide solution, binding to DNA, RNA, and protein was found. This observation may allow a new approach to the problem of the biologic significance of hydrocarbon-nucleic acid reactions.

Having established that the hydrocarbons, like all the other carcinogenic agents which have been discussed, react in the cell with both nucleic acids and proteins, we again have the problem of the vital site for the initiation of tumors. Considering the quantitative aspect of hydrocarbon carcinogenesis, it is clear that if this is a consequence of the firm binding of the hydrocarbon to any cell component, rather than to a transient physicochemical interaction which is broken down during isolation, then it must result from very low levels of reaction. The binding of hydrocarbon to skin DNA found by Brookes and Lawley (15) following application of 1 μmole of hydrocarbon/mouse was in the range 0.1–1.0 molecule of hydrocarbon per DNA molecule of molecular weight 6.7 × 10^6, while for protein the binding represented 0.01–0.03 molecule of hydrocarbon/protein molecule of molecular weight 1 × 10^5. Heidelberger (39), discussing the extent of binding to the specific fraction of mouse-skin soluble protein, to which carcinogenic hydrocarbons were found to be bound, calculates that 1 in every 170 protein molecules in this fraction has a hydrocarbon molecule bound to it. These levels of protein binding do not seem consistent with the protein deletion theory as discussed by the Miller’s (61) which is largely based on the observation of Sorof and Cohen (72) that hepatomas induced by chemical carcinogens are lacking measurable amounts of protein. Such a gross deletion of protein would seem more likely to result from a reaction at the level of the genome resulting in inactivation of that section of the DNA coding for the missing protein. The theory of Pitot and Heidelberger (64), based on the work of Jacob and Monod (45), was designed to overcome some of the objections to the protein deletion theory by assuming that

![Chart 5](chart5.png)

**Chart 5.** The extent of binding to mouse skin DNA found for a series of hydrocarbons, plotted against the Iball index of carcinogenic activity. ▲, naphthalene and benzo(e)pyrene; ◇, Dibenzo(a, c)anthracene; ●, benzo(a)pyrene; ○, dibenz(a, h)anthracene; ■, 3-methylcholanthrene; □, 7,12-dimethylbenz(a)anthracene; △, benz(a)anthracene.

The extent of binding to mouse skin DNA found for a series of hydrocarbons, plotted against the Iball index of carcinogenic activity. ▲, naphthalene and benzo(e)pyrene; ◇, Dibenzo(a, c)anthracene; ●, benzo(a)pyrene; ○, dibenz(a, h)anthracene; ■, 3-methylcholanthrene; □, 7,12-dimethylbenz(a)anthracene; △, benz(a)anthracene.
the protein which binds the carcinogen is the repressor protein of a regulatory circuit controlling cell division. But again it is not clear why this scheme should lead to a gross loss of protein from the resulting tumor cell, since the repressor which is presumed to be inactivated by the hydrocarbon, and the synthesis of which is thus prevented, could only represent a very small fraction of the total cellular protein. The theory also requires a highly selective reaction of the hydrocarbon with the particular repressor involved in the control of cell division. The significance of these ideas to carcinogenesis must be in doubt until there is a clear demonstration that control mechanism in mammalian cells resemble those in bacteria. A full discussion of these problems has recently been published by Potter (65).

General Discussion

I have tried to emphasize in this review the quantitative aspect of carcinogen cell interaction and the data discussed are summarized in Table 1.

It is perhaps worth emphasizing that those papers which stress the significance of nucleic acid interaction (9, 11, 14, 15) also report protein binding at a level similar to that reported by others who stress the significance of this reaction. It is the importance and not the occurrence of protein binding which is in dispute.

The results presented in Table 1 inevitably must revive the somatic mutation theory which originated with Boveri (7) and which has been strongly criticized in the past few years (16, 70). The criticisms have largely been concerned with the failure to demonstrate mutation by such well-tested carcinogens as the hydrocarbons on the one hand, and the failure to obtain tumors with such proven mutagens as nitrous acid and formaldehyde on the other. However, the tests of mutagenicity and carcinogenicity are of necessity not carried out with the same organism. A frequency of mutation of 1 in $10^4$, which is readily detected in microorganisms is well outside the scope of tests in mice, and the mutation leading to neoplasia may well be of this frequency. It may be relevant that the frequency of transformation of tissue culture cells by a tumor virus is at best of the order of 1 in $10^4$ (78).

Perhaps of greater significance is the idea that the molecular event leading to an efficient mutation in bacteria or phage may not result in a mutation in higher organisms while a gross deletion causing a significant alteration in the functioning of an animal cell may be lethal in a microorganism. Ideas on this subject have been reviewed by Kondo (48) who emphasizes that differences in mechanisms of repairing and tolerating premutational DNA damage shown by different organisms may be of prime importance in the establishment of mutations. He also shows that the resistance to killing by radiation increases greatly with increasing genome complexity and that higher plants, for example, have a high tolerance for mutations involving chromosome aberrations. He concludes that there are mutagenic factors differing with genome complexity, in addition to those common to all living things.

Those compounds such as the nitrosamines and alkylating agents which have been shown to be mutagenic in microorganisms as well as carcinogenic may be capable of producing both low severity and high severity DNA damage.

The idea that chemical carcinogenesis is a 2-stage process

### Table 1

<table>
<thead>
<tr>
<th>Agent used</th>
<th>ROUTE OF ADMINISTRATION AND DOSE (mg/kg)</th>
<th>TIME OF TREATMENT (hr)</th>
<th>SOURCE OF TISSUE EXAMINED</th>
<th>EXTENT OF REACTION OF CELLULAR MACROMOLECULE (mole carcinogen/mole NADP or protein)</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfur mustard</td>
<td>0.2; i.p.,</td>
<td>0.5</td>
<td>Ascites tumor cells in mice</td>
<td>DNA (Mol. Wt. $6.7 \times 10^6$) 0.05, RNA (Mol. Wt. $2 \times 10^6$) 0.015, Protein (Mol. Wt. $1 \times 10^5$) 0.0075</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>2.0; i.p.</td>
<td>0.5</td>
<td>Ascites tumor cells in mice</td>
<td>Not determined, DNA 0.6, RNA 0.19, Protein 0.0075</td>
<td>11</td>
</tr>
<tr>
<td>Diethylnitrosamine</td>
<td>200; i.p.</td>
<td>24</td>
<td>Liver of rat</td>
<td>DNA Not determined, RNA 16, Protein Not determined</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>200; i.p.</td>
<td>24</td>
<td>Kidney of rat</td>
<td>DNA Not determined, RNA 3, Protein Not determined</td>
<td>56</td>
</tr>
<tr>
<td>β-Propiolactone</td>
<td>7; skin painting</td>
<td>24</td>
<td>Mouse skin</td>
<td>DNA 0.17, RNA 0.10, Protein 0.005</td>
<td>Brookes and Lawley (unpublished work)</td>
</tr>
<tr>
<td>Dimethylaminoazobenzene</td>
<td>150; i.p.</td>
<td>16</td>
<td>Liver of rat</td>
<td>DNA 0.15, RNA 0.34, Protein 0.07</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>5.5; stomach tube</td>
<td>16</td>
<td>Liver of rat</td>
<td>DNA Not determined, RNA 0.01, Protein 0.009</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>5.5; stomach tube</td>
<td>16</td>
<td>Liver of cotton rat</td>
<td>DNA Not determined, RNA 0.003, Protein 0.001</td>
<td>59</td>
</tr>
<tr>
<td>2-Acetylaminofluorene</td>
<td>22; stomach tube</td>
<td>48</td>
<td>Liver of rat</td>
<td>DNA Not determined, RNA 0.05, Protein 0.003</td>
<td>58</td>
</tr>
<tr>
<td>7,12-Dimethylbenz(a)-anthracene</td>
<td>1.5; skin painting</td>
<td>22</td>
<td>Mouse skin</td>
<td>DNA 0.09, RNA 0.013, Protein 0.001</td>
<td>15</td>
</tr>
<tr>
<td>Benzo(a)pyrene</td>
<td>4; skin painting</td>
<td>42</td>
<td>Mouse skin</td>
<td>DNA 0.075, RNA 0.1, Protein 0.0045</td>
<td>15</td>
</tr>
</tbody>
</table>

* NA, nucleic acid.
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consisting of initiation and promotion we owe to the work of Berenblum, Rous, Rusch, and others [see review by Boutwell (6)]. The initiation stage which is irreversible (3) is most easily thought of as skin to mutation while the promotion phase which Boutwell (6) showed to be reversible might possibly involve changes at the level of cellular enzyme systems. In the past when several rival theories have each received strong support, the truth has ultimately been shown to embrace all points of view. Is it not possible that this may be so for the obviously complex process of carcinogenesis?

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Peter Brookes


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