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BIOCHEMICAL PHARMACOLOGY OF THE INTESTINAL FLORA

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It is now recognized that some of the metabolites of exogenous compounds that are observed in the excreta of animals reflect the activity of the intestinal bacteria. One recent review has stressed the chemical characterization of these bacterial reactions (1). Another has called attention to possible clinical considerations of drug metabolism by the flora (2). This review, however, emphasizes recent studies that suggest how reactions by the intestinal microflora may have implications for determining the effect of exogenous compounds on the animal host. Thus attention is confined to those metabolic transformations by the flora that have implication either for drug action or toxicity and for chemical carcinogenesis.

THE STUDY OF METABOLIC REACTIONS ATTRIBUTED TO THE FLORA

The nature of the normal flora must be appreciated in order to gain some perspective on whether results obtained in the laboratory are likely to be applicable to the physiology of the flora in the animal host.

The intestinal bacteria are part of a complex ecosystem; the species comprising the predominant flora are adapted to rigid conditions of pH and oxygen tension and may require complex growth factors. Thus the natural environment of the flora may be difficult to simulate in laboratory media. Nevertheless, methods have been devised to prepare culture media under conditions of oxygen exclusion which enable the microorganisms of the gut to be cultivated. With proper methods (3, 4), the bacteria enumerated under the microscope in a sample of colon contents or feces (approximately 10^{11} bacteria per gram) can now be reproduced almost quantitatively in culture. Since there are at least 400 different species of bacteria in the human intestinal tract at any time (5), it is easy to cultivate some bacteria from the gastrointestinal tract and to study their metabolic capabilities. The exquisite requirements of the predominant normal flora suggest, however, that greater physiological

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significance should be attributed to those reactions that can be demonstrated with bacteria treated under conditions that simulate their normal environment. A minimal requirement is that bacteria be removed promptly from the gut of a sacrificed animal and cultured under anaerobic conditions. Unless these physiological limitations are considered, the bacterial reactions studied in the laboratory are not apt to represent those of the normal flora. Rather they may be reactions of the aerobic bacteria which comprise less than 0.1% of the normal flora. The activity of the aerobic flora is apt to be of little quantitative significance to the animal host.

Even observance of the above precautions may not assure that the cultivated bacteria and their reactions are representative of those that have significant activity in the host. Histological studies of the bacteria lining the intestinal tract of rodents have shown a well-developed organization of the different bacterial species in relation to the various components of the gut wall (6). These studies suggest that the gut bacteria are adapted very precisely to a defined microenvironment within the gut. Thus, even when the metabolism of appropriate bacteria is studied under laboratory conditions which assure total recovery of bacteria, the results may still not elucidate the role of the flora as it functions within the constraints of its normal ecosystem. This possible problem is suggested by the result of a study comparing the bacterial metabolism of caffeic acid in culture with that carried out by the same bacterium within the animal host. Caffeic acid, a component of the vegetable matter in the diet, was selected for this study because the metabolic reactions required for its transformation in vivo can be demonstrated in cultures of individual bacteria isolated from the feces. Studies in germfree rats indicate that mammalian metabolism plays no part in these reactions. When germfree rats were associated with bacteria capable of various reactions in the caffeic acid pathway, the rat gained the capacity to make these transformations. It was found, however, that the reactions conferred on the rat by a given bacterial association were not necessarily those which the bacteria had catalyzed in culture (7). Thus there is no assurance that a bacterial reaction demonstrated in culture will occur in the animal simply because that bacteria is lodged in the gastrointestinal tract of an experimental animal.

Another problem is that of applying results obtained in one animal species to that which might occur in another. The usual problem is that the implications for man are sought in data obtained from experimental animals. The rat, which is a convenient source of experimental data, has a flora in the forestomach and in the upper small intestine and thus differs from the human whose upper gastrointestinal flora ordinarily is negligible (8). Anatomical difference in the location of the flora may be important since drugs absorbed high in the gastrointestinal tract may undergo metabolism in the rat, whereas in the human, metabolism by the flora ordinarily might not occur unless the drug reaches the colon. In addition, pharmacokinetic characteristics such as the biliary excretion of drugs may differ from one species to another (9) and thus a drug may gain access to the bacteria of the gut in one animal species but not in another.

When a compound is administered to both germfree and conventional animals and its metabolites are found only in the urine or feces of the conventional animals, the flora can be assigned a tentative role in the metabolic pathway. This evidence

is not necessarily proof that the flora is involved in the metabolic pathway since there are other reasons for different responses between germfree and conventional animals. However, when the reaction missing in the germfree animal can be demonstrated in cultures of intestinal bacteria, the tentative assignment appears more reasonable. A decrease in the formation of a metabolite when the conventional animal is treated simultaneously with an antibiotic is further presumptive evidence for the participation of the flora in a metabolic pathway. However, it must be remembered that antibiotics such as the aminoglycosides and sulfas can make alterations in the concentration of a compound by actions not related to their antibacterial activity (10). In addition, antibiotics can cause diarrhea and thus might alter the extent of the formation of a metabolite simply by increasing the intestinal transit time of the parent compound rather than by diminishing the metabolic activity of the flora.

Adventitious alterations in physiology may occur after surgical procedures that are intended either to increase the flora through the creation of blind loops or to decrease it by cecectomy. Thus, changes in metabolism noted after these procedures are not necessarily a consequence simply of an alteration of the flora.

THE FLORA AND DRUG EFFICACY

Sulfa Derivatives

Prontosil and neoprontosil, the earliest sulfa drugs to be used therapeutically, become bacteriostatic only when sulfanilamide is released by reduction of the azo bond which links the blocking group to the N^4 position of the sulfa moiety (11). Although azo bond reduction can be mediated by liver preparations (12), the intestinal flora apparently plays a principal role in this critical reaction because the amount of sulfonilamide in the urine of rats fed either prontosil or neoprontosil is greatly decreased when additional antibiotics are given to suppress the intestinal flora (13). Thus it seems likely that the flora played a critical role in the activation of these early antibacterial agents.

Sulfasalazine (salicylazosulfapyridine, Azulfidine®) is a sulfa drug blocked at the N^4 position which retains a position in modern therapy because it is effective in preventing recurrences of ulcerative colitis and in treating moderately severe granulomatous disease of the bowel (14). The azo bond of this drug joins the N^4 position of sulfapyridine and the amino nitrogen of 5-aminosalicylate. After oral administration of sulfasalazine, only sulfapyridine, 5-aminosalicylate, and their respective metabolites are found in the urine and feces of conventional rats, indicating that reduction of the azo bond has been complete. In contrast, the excreta of germfree rats receiving sulfasalazine contain only sulfasalazine itself; metabolites that would indicate reduction of the azo bond are not detectable (15). Moreover, administration of neomycin, which is known to diminish bowel flora, decreases the reduction of sulfasalazine in conventional animals. Consistent with the obligate role of the flora in the reduction of the azo bond of sulfasalazine is the observation that cultures of many bacteria characteristic of those in the gastrointestinal tract carry out the reaction missing in the germfree rat. When only one strain capable of azo

bond reduction in vitro is associated with a germfree rat, the rat gains the capacity to reduce sulfasalazine (15). Thus it seems clear that the reaction of azo bond reduction which initiates the metabolism of sulfasalazine in the rat is mediated by the intestinal flora.

In normal human volunteers only a small fraction of sulfasalazine is excreted in the urine (16, 17). As in the rat, the drug appears to be absorbed in the small intestine and then is returned to the intestine via the bile. Bacteria in the colon reduce the azo bond and release sulfapyridine and 5-aminosalicylate. Sulfapyridine is readily absorbed and may undergo further metabolism (acetylation and hydroxylation) before elimination in the urine. 5-Aminosalicylate, however, is poorly absorbed from the colon and is more apt to be excreted in the feces (16, 18).

When 5-aminosalicylate is administered orally to rats it is readily excreted in the urine. Thus, oral 5-aminosalicylate appears to be absorbed from the upper gastrointestinal tract and is readily excreted in the urine. When administered orally in the form of sulfasalazine, however, 5-aminosalicylate is distributed to the colonic contents and is excreted in the feces (18). 5-Aminosalicylate also tends to be excreted in the feces when sulfasalazine is administered parenterally, whereas it is readily eliminated in the urine when given parenterally as 5-aminosalicylate. These different distribution patterns suggest that sulfasalazine may serve simply as a means of increasing the concentration of 5-aminosalicylate and hence also of sulfapyridine in the colon. Thus it has been suggested that sulfasalazine may exert its therapeutic effect by serving as a vehicle for the delivery of either or both of its component molecules to the colon where they might act topically (15, 19). This speculation becomes more justifiable in view of a recent report that prostaglandins, whose synthesis is inhibited by either 5-aminosalicylate or sulfasalazine, appear to be increased in the feces of patients with active ulcerative colitis (20).

Some of the failures to achieve therapeutic benefit with sulfasalazine may reflect variations in the concentration of sulfasalazine or of one of its possibly active metabolites in the colon. Such variations in turn might be a manifestation either of the metabolic capacity of the flora or of the intestinal transit time that determines the extent of contact between the drug and the flora.

Lactulose

Lactulose, (4-β-D-galactopyranosyl-D-fructose) is effective in treating portal-systemic encephalopathy, a manifestation of liver insufficiency which is believed to be caused by an elevation of the serum ammonia concentration. The nonabsorbable lactulose is hydrolyzed and metabolized by the flora, resulting in the formation of organic acids such as lactic and acetic acids. These acids lower the pH of the intestinal contents. At the lower pH, ammonia and amines are protonated and their absorption is retarded. In addition some believe that the organic acids derived from lactulose can cause an osmotic diarrhea which further enhances the elimination of the neurotoxic ammonia and amines and decreases their concentration in blood and brain. This explanation for the action of lactulose finds support from observations that lactulose is hydrolyzed by bacteria but not by enzymes from the intestinal wall (21) and by the observation that the clinical effectiveness of the drug is accompanied by a decrease in fecal pH (22).

Cathartics

The action of certain cathartics depends on metabolic activation that is probably mediated by the flora. Cascara and senna are glycosides of anthraquinones. The anthraquinones stimulate peristalsis only when released from the parent compounds in reactions that the available evidence suggests might be mediated by the microflora (23). The role of the flora is somewhat better defined for the cathartic action of 4,4'-dihydroxydiphenyl(pyridyl-2)methane which stimulates peristalsis by causing the accumulation of fluid in the colon. The sulfate ester of this compound is not hydrolyzed by the mucosa and is not active until the ester is hydrolyzed, presumably by the intestinal bacteria. The sulfate ester causes more prompt laxative action than the free drug (24), presumably because the polar sulfate derivative is not absorbed and reaches the lower level bowel promptly where it is hydrolyzed by the flora to release the active compound. When the free drug is taken, however, it does not reach the lower bowel so promptly because it may be absorbed, conjugated to glucuronic acid, and only then be excreted in the bile. Free drug forms in the lower bowel when the glucuronide is hydrolyzed by the flora. The pathway of the free drug to the bowel is thus more circuitous than that of its sulfate ester.

THE FLORA AND THE TOXICITY OF DRUGS AND OTHER EXOGENOUS COMPOUNDS

L-Dopa

The activities of both mammalian and bacterial enzymes are required to form at least two of the many metabolites of L-dopa that are found in the urine. These metabolites, *m*-tyramine and *m*-hydroxyphenylacetic acid, are greatly decreased in the urines of parkinsonian patients taking L-dopa when neomycin is also administered (25, 26); both compounds are found in the urines of conventional rats but not of germfree rats given L-dopa (27). Dehydroxylation at the 4-position of the catechol ring, in the one instance of dopamine and in the other of 3,4-dihydroxyphenylacetic acid, is required to form these metabolites. Neither of these reactions can be demonstrated in germfree rats but both can be demonstrated when the appropriate substrate is incubated anaerobically with rat cecal contents (27). Thus the flora seems to have an obligate role in the formation of these trace metabolites of L-dopa.

Sandler has called attention to the possible clinical importance of trace metabolites such as these, and his suggestion (26) gains support from an experiment in which L-dopa was administered to parkinsonian patients in a formulation that retarded its absorption. When L-dopa was given in this form, there was no change in neurological benefit but there was an intolerable increase in gastrointestinal side effects together with a simultaneous alteration in the concentrations of trace metabolites in the urine. *m*-Hydroxyphenylacetic acid was increased, while 3,4-dihydroxyphenylacetic and homovanillic acids were decreased (28). These changes are consistent with a greater effect of the flora on the metabolism of L-dopa.

Metabolic alterations suggesting a decreased effect of the flora occur when a rat is deprived of food for 4 days. Under these circumstances the quantity of flora in the lower bowel is decreased. When L-dopa is then administered in a small amount

of food, the urinary elimination of *m*-hydroxyphenylacetic acid is diminished and the metabolites not dependent on the flora tend to increase (29). Since most of an oral dose of L-dopa does not reach the systemic circulation, a great deal of the drug may remain in the gut where it is available to the flora for the formation of these possibly active trace metabolites.

Mercury

It has been suggested that the flora participates in some of the interconversion of inorganic and organic mercury that may occur in the host. This may have some significance since methylmercury with its more facile entry into the central nervous system is believed to be more neurotoxic than inorganic mercury.

Mercury is eliminated in inorganic form in the feces even when administered to rats in the form of methylmercury (30). This observation seems incompatible with the fact that the bile of these rats contains high concentrations of methylmercury cysteine and little inorganic mercury (30). The apparent incompatibility could be reconciled if it were demonstrated that the flora was capable of degrading methylmercury cysteine.

There is a suggestion that the flora can synthesize methylmercury from inorganic mercury. Suspensions of cecal contents carry out this synthesis both aerobically and anaerobically and the reaction appears to be bacterially mediated since it is diminished by filtering or autoclaving the suspension or by treating it with antibiotics (31). A physiological role for the flora in organifying mercury is compatible with the observation that rats with blind jejunal loops get more prominent neurological symptoms and have higher tissue concentrations of methylmercury than control rats after 3 weeks of inorganic mercury administration. The difference imposed by the creation of a blind loop, however, may not be due solely to increased methylation by the bacterial flora since inorganic as well as organic mercury is highly concentrated in the rats with blind loops (32). It is likely therefore that the animals with the blind loops may simply have a decreased capacity to eliminate the acute mercury burden.

A role for the flora in both synthesis and degradation of methylmercury is suggested by the observation that methylmercury is synthesized during prolonged incubation with cecal contents only to disappear when the incubation is more prolonged (31). However, when the disposition of methylmercury was compared in germfree and conventional rats, the quantity of inorganic mercury in the feces and cecal contents was not different (33). Thus it is still uncertain whether the flora has a meaningful role in mercury metabolism.

Compounds Containing the Nitro Group

Metabolic transformations of the nitro group may explain toxicity that is caused by certain compounds containing this functional group. Among the examples that can be cited are methemoglobinemia from nitrobenzene (34), carcinogenesis from nitrofurazone (35), and even aplastic anemia from chloramphenicol (36). It has been assumed that biological reduction of the nitro group proceeds through nitroso

(–N=O) and hydroxylamine (–NHOH) intermediates (37) and it has been further assumed that toxicity arises in some cases from these reactive metabolites (38).

Rats fed *p*-nitrobenzoic acid show substantial amounts of *p*-aminobenzoic acid in their urine; since *p*-aminobenzoic acid is so readily quantified, this transformation has served as the prototype of the biological reduction of the nitro group which presumably occurs with other compounds of greater pharmacological and toxicological interest. In vitro the conversion of *p*-nitrobenzoic acid to *p*-aminobenzoic can be demonstrated either with bacteria isolated from the intestinal tract (39, 40) or with a preparation using the cytochrome P450 system of rat liver (41). The physiological significance of nitro group reduction by the liver preparation has been questioned since it requires the unphysiological exclusion of oxygen and thus differs from the usual cytochrome P450–dependent reactions of drug metabolism (37, 42).

The conversion of *p*-nitrobenzoic acid to *p*-aminobenzoic acid that occurs in vivo is probably due to the flora since the extent of this transformation both in the animal and in isolated intestinal contents is decreased by antibiotic treatment (40). More definitive evidence of an obligate role for the flora in this reaction comes from the observation that the conversion of *p*-nitrobenzoic acid to *p*-aminobenzoic acid is negligible in germfree rats (42) but is progressively increased as these rats are selectively associated with intestinal bacteria having increasingly greater capacity for this reaction in culture (42). It had been observed that rats treated with phenobarbital or DDT showed no increase in their capacity to reduce orally administered *p*-nitrobenzoic acid although this activity was significantly induced in preparations of their liver microsomes (43). These results are puzzling if it is assumed that nitro group reduction is mediated by the liver but are perfectly compatible with nitro group reduction by the flora.

Metabolites arising from reduction of the nitro group of *p*-nitrobenzenesulfonamide are abundant in the urine of conventional rats but are negligible in that of germfree rats (42). Attributing this reaction to the flora offers an explanation for another incompatibility between mammalian enzyme activity observed in vitro and the occurrence in vivo of the reaction attributed to that enzyme. *p*-Nitrobenzenesulfonamide can be reduced by xanthine oxidase of rat liver (44) and the activity of this molybdenum-containing enzyme can be greatly depleted by prolonged feeding of sodium tungstate. The reduction of orally administered *p*-nitrobenzenesulfonamide is only slightly decreased, however, in rats whose liver xanthine oxidase is greatly depleted by tungstate feeding (44).

The obligate role of the flora in nitro group reduction has its counterpart in studies demonstrating that toxicity of a compound depends on the presence of the flora. Nitrobenzene (200 mg/kg intraperitoneally) causes 30–40% methemoglobinemia within 1 to 2 hr in normal Sprague Dawley rats. This response to nitrobenzene is negligible, however, in germfree rats and in rats previously given antibiotics to suppress their bacterial flora (34).

The aplastic anemia caused by chloramphenicol is a practical problem which has been suggested to be a possible consequence of drug metabolism by the flora (36). Metabolites of chloramphenicol having their nitro group replaced by amines are excreted in rat and human urine and achieve particularly high concentrations in the

feces of both rat and dog (45). In addition, amine derivatives are prominent in the urine of a rat if chloramphenicol is instilled into a surgically created cecal loop but not if instilled into a jejunal loop which contains fewer bacteria. Thus reduction of the nitro group in chloramphenicol appears to be mediated by the flora (46).

Citing the absence of recorded cases of aplastic anemia after chloramphenicol was given solely by the parenteral route, Holt proposed in 1967 (36) that the flora might be responsible for metabolizing chloramphenicol to a hypothetical compound which more directly mediates this rare toxic reaction. Contact of the drug with the flora could be greatly decreased by parenteral administration because the biliary excretion of chloramphenicol appears to be considerably less in the human than in the dog or guinea pig (45). Furthermore, although human feces are capable of reducing the nitro group of chloramphenicol, little of the amine metabolite is ordinarily found in feces of humans receiving the drug (46). Thus there are data to suggest that the parenteral administration of chloramphenicol might indeed limit its access to the intestine and thus to the bacteria which possibly might form a toxic metabolite.

What then is the evidence that the likelihood of aplastic anemia in the patient taking chloramphenicol may be determined by the route of administration? Robert J. Hans (personal communication) has examined the world's literature for case reports on aplastic anemia and has attempted to gather complete details of each case said to involve exclusively parenteral administration. His findings on reviewing approximately 500 cases support Holt's impression. Aplastic anemia has been reported in only four patients whose exposure to chloramphenicol was exclusively by the parenteral route (47–50). Furthermore, Hans points out that each of the patients of this small group had another possible factor, e.g. influenza, old age, or exposure to another drug causing aplastic anemia, which may be implicated instead of chloramphenicol for the aplastic anemia.

The significance of the apparent low incidence of aplastic anemia after parenteral chloramphenicol can be determined only if additional information is available. That information is the relative frequency of exposure in this population to chloramphenicol given exclusively by the parenteral route. Only with these data can the risk of aplastic anemia following exclusively parenteral administration be shown to differ from the risk when oral chloramphenicol is given. It seems unlikely that an estimate of relative exposure, which is critical for Holt's argument, can be obtained for the population of patients that contributes to a cohort so poorly defined as the world's medical literature. The relationship between the route of administration and the incidence of aplastic anemia due to chloramphenicol could in principle, however, be examined in a more defined cohort. The study of patients dying of aplastic anemia in California during an 18-month period (50) might be suitable for establishing this relationship. Unfortunately for the analysis, however, the numbers available are too small to test Holt's hypothesis. The California study identified only ten instances of aplastic anemia in which chloramphenicol could be implicated. (One was that of an 11-year-old girl who had had parenteral chloramphenicol.)

There are other examples which suggest that biological reduction is essential for certain toxic reactions of compounds containing the nitro group. Disruption of circular DNA by the carcinogen nitrofurazone, a reaction possibly related to its

action as a carcinogen, depends on the presence of bacterial nitroreductase (51). The mutagenicity of metronidazole for *Salmonella typhimurium* strain TA100 also depends on nitroreductase activity, either that in the salmonella tester strain itself or that isolated from liver (52). Reduction of the nitro group may be essential for the activity of metronidazole which is bacteriocidal for anaerobic but not aerobic bacteria (43). These additional examples of nitro group reduction in mediating selective toxicity suggest that the toxicological importance of nitro group reduction by the flora may extend beyond those compounds mentioned earlier.

The Flora and Detoxification Reactions of the Liver

Consideration is now given to the possible significance of metabolic reactions of the flora as they relate to the "detoxification reactions," particularly those occurring in the liver. In a sense reactions of the flora can be viewed as complementary or perhaps antagonistic to those of the liver. The cytochrome P450–dependent reactions of the liver microsomes require molecular oxygen while those of the flora may be inhibited by oxygen. The liver tends to oxidize compounds, and the flora to reduce them. With some compounds the flora can nullify an oxidative reaction carried out by the liver. Similarly the flora can hydrolyze glucuronides, sulfate esters, and acylamides, and thus reverse the conjugation reactions carried out in the liver.

The deconjugation of a glucuronide may be essential for the enterohepatic circulation of a drug because the water-soluble glucuronide group prevents the drug conjugate from being reabsorbed. When the glucuronide of diethylstilbesterol is instilled in the duodenum, for example, and deconjugation is inhibited either with antibiotics to suppress the flora or with a specific inhibitor of β-glucuronidase, the absorption of diethylstilbesterol is diminished (54). When rats are treated with lincomycin, the metabolism of diethylstilbesterol is altered in a manner consistent with an interruption of the enterohepatic circulation but the estrogenic effect measured by uterine size remains the same (55). [Several possible reasons have been advanced for this dissociation between the pharmacokinetic and the pharmacodynamic properties of diethylstilbesterol (55).] Thus even for this well-studied drug, the importance of the flora in making significant changes of drug elimination by altering deconjugation reactions remains unproved. Less information is available on whether there are significant effects of the flora on the actions of other drugs, morphine (56) and indomethacin (57), which are excreted conjugated to glucuronic acid in the bile.

Amine and amide-containing carcinogens are N-hydroxylated by the cytochrome P450 system of the liver in the obligatory first step of the metabolic sequence leading to the formation of the electrophilic metabolites which react with cellular macromolecules to initiate carcinogenesis (58). N-Dehydroxylation, the reaction which reverses this first step toward the formation of the ultimate carcinogen, is mediated by the flora.

The possible interplay between N-hydroxylation in the liver and N-dehydroxylation by the flora was initially suggested by studies with the carcinogen fluorenylacetamide. N-Hydroxyfluorenylacetamide is dehydroxylated by cultures of *Escherichia coli* strain K32 and by aerobic preparations of cecal contents of conven-

tional but not of germfree rats (59). The progressive increase in the rate of N-dehydroxylation observed in these aerobic experiments can be attributed to the development of a more physiological anaerobic environment as oxygen is consumed in the incubating mixture by facultative bacteria.

Studies with the related carcinogen N-hydroxyacetylaminobiphenyl demonstrated that the rate of the N-dehydroxylation reaction was enhanced when anaerobic bacteria were maintained under anaerobic conditions (60). These studies also showed that N-hydroxyacetylaminobiphenyl is N-dehydroxylated more rapidly in cecal than in stomach contents. The relative rates of these reactions may bear on the different susceptibility of different parts of the gut to carcinogenesis with N-hydroxyacetylaminobiphenyl. Rats fed this compound get tumors of the forestomach but not elsewhere in the gastrointestinal tract (61). Since there is no N-dehydroxylating activity anywhere in the gut wall (60), the susceptibility of the stomach might be related to the nature of its flora. Lactobacillus is the predominant flora at this site and has only weak capacity for this possibly protective dehydroxylating reaction (60).

N-Hydroxyfluorenylacetamide and N-hydroxyacetylaminobiphenyl both undergo glucuronidation at the N-hydroxyl group. N-Hydroxyfluorenylacetamide is the stronger carcinogen, and its glucuronide is much more reactive with nucleic acids. Thus it has been suggested that these glucuronide derivatives may act as weak ultimate carcinogens (62). These glucuronides are recovered from the cecums of germfree rats but are virtually absent in the cecal contents of conventional rats fed either fluorenylacetamide (63) or N-hydroxyacetamidobiphenyl (60). The glucuronides survive also in the cecal contents and feces of germfree but not of conventional rats after administration either of N-hydroxyfluorenylacetamide (64) or N-hydroxylacetamidobiphenyl (60). It is unclear, however, whether these metabolic contributions by the flora influence the carcinogenic action of these compounds.

Indeed although our knowledge of reactions of the flora with *possible* toxicological consequences continues to expand, there are only a few instances where the correlation between metabolism and toxic consequences has been demonstrated. In this connection one must mention cycasin, which is carcinogenic for conventional but not germfree rats, because the flora provides the glucosidase necessary for its activation (65).

The Further Study of This Problem

How is one to study further the possible toxicological implications of metabolic reactions carried out by the flora? It is not sufficient simply to indicate that the flora is responsible for possibly toxic reactions that occur in the whole animal. One would like evidence that toxic metabolites arise in the flora and then reach the organs that manifest toxicity. Unfortunately, many reactive toxic compounds formed by metabolic activation, such as those involved in carcinogenesis (58), are unstable. Unstable compounds are not readily detected by the methods outlined earlier in this review. These methods help to distinguish between mammalian and bacterial reactions. They are applicable, however, only to compounds that are stable enough to be administered to an animal and serve only to identify metabolites that are stable

enough to withstand isolation from biological fluids. Thus the existence of labile, reactive intermediates, which may be responsible for toxicity, can usually only be inferred from reaction sequences compatible with the formation of isolable metabolites. It would be desirable to have a system for detecting the toxic, labile, metabolic intermediates themselves.

For this reason, interest has recently turned to a specific group of histidine auxotrophs of *Salmonella typhimurium* designed by Ames. These mutants are extremely sensitive to mutagenesis which is readily detected by reversion of the auxotrophs to histidine independence. Mutagenic activity can then be quantified by enumerating the bacteria which form colonies on histidine-deficient petri plates (66). The utility of the Ames mutants for detecting reactive compounds can be improved if microsomal preparations from rat liver and cofactors are incorporated into the bacterial test system. The addition of the mammalian enzyme system provides metabolic activation of the test compound, the capacity for which may be lacking in the salmonella testor strain. It has been noted that carcinogenic compounds are very likely to be reactive in the test system, particularly when fortified with the liver preparation whereas noncarcinogens are unlikely to cause a mutagenic response (67). A number of chemical carcinogens, however, cause no response with any of the testor strains. The weak or absent response to hydrazine compounds, particularly the colon carcinogen 1,2-dimethylhydrazine (67), is noteworthy.

The failure of the in vitro salmonella test systems currently available to respond to known chemical carcinogens may have a number of explanations. One is that some carcinogens may require metabolic activation provided by enzymes of organs other than the liver. The host-mediated assay provides an approach to this problem by combining the metabolic activity of the whole animal with the detection system of the Ames tester strains. This concept has been applied by Legator to the mouse whose peritoneum is seeded with a culture of the tester strain. After a compound is administered, the peritoneal bacteria are recovered and the revertants enumerated (68).

Results from this host-mediated assay initially suggested the possibility that a mutagen might arise from the antischistosomal compound 4-isothiocyanato-4'-nitrodiphenylamine at a site other than the liver. This antischistosomal compound is not mutagenic in vitro to Ames tester strain TA100 even in the presence of microsomal preparations, but mutagenesis occurs with the tester strain in the mouse peritoneum (69). Metabolic activity by the flora may explain this observation since mutagenic activity in mouse urine after feeding 4-isothiocyanato-4'-nitrodiphenylamine is markedly decreased by prior treatment with succinylsulfathiazole. Since the sulfa drug does not interfere with antischistosomal activity it suggests that therapeutic activity might be retained and mutagenic activity suppressed by controlling the metabolic activity of the flora.

Another host-mediated assay makes use of tester strain TA1538 (a frameshift mutant) which, after it is fed to otherwise germfree rats will colonize their gastrointestinal tracts. These bacteria can be maintained in a stable association with the rat for at least 7 months (70) and achieves concentrations of greater than 10^7 per gram in the stomach and greater than 10^8 per gram in the lower bowel and feces. The

normally low number of revertants passed in the feces was greatly increased when known carcinogens were administered to these rats. Structurally related compounds without carcinogenic activity failed to increase the number of revertants in the feces (70). Although the concentration of revertants in the feces of these animals was correlated with that in the lower bowel, it is not clear that revertants actually form in the colon. Revertants may form higher in the gut, in the stomach for example, and then be transported to the lower bowel. Although the site of revertant formation is unclear, the number of revertants found in the feces is clearly related to the dose of 2-nitrofluorene (71).

Selected additional strains characteristic of the normal gastrointestinal flora can be associated in the axenic rat along with strain TA1538. In the presence of the additional strains (*Lactobacillus plantarum* and *Bacteroides vulgatus*), the number of revertants formed in the response to the carcinogen 2-nitrofluorene is blunted. It is uncertain whether the decreased response in the presence of the additional flora is due to metabolic activity of the added bacteria (culture of *B. vulgatus* actively reduce the nitro group of 2-nitrofluorene in vitro) or is simply the result of the displacement of the salmonella from the site in the gut where they are at risk for mutagenesis (72). Although strain TA1538 remains in the gut when up to three additional components of the flora are added, it cannot be recovered from the feces when the selectively associated rat is allowed to leave the axenic environment and becomes "conventionalized" (72). Thus controlled conditions are required if the tester strain is to remain in the colon as a means of detecting mutagenic activity.

CARCINOGENESIS AND THE FLORA

The incidence of cancer varies among different population groups but a high incidence of certain tumors tends to occur where the diet contains large amounts of meat or animal fat (73). Colon cancer in particular has been related to the consumption of meat (73) and animal fat (74) and the possible pathogenic mechanisms responsible for this correlation are under investigation. A correlation between the microbial composition of the feces and the epidemiology of colon cancer has been suggested (75), and the evidence relating to this question has recently been reviewed (5). It has been shown that bacterial enzymes in the feces, possibly those relating to chemical carcinogenesis, are altered by changes in the diet. A meat diet increases β-glucuronidase in the feces of man (76) and rat (77) and increases enzymes such as azo- and nitro reductase in the feces of the rat (77).

Particular attention has focused on the suggestion that intestinal bacteria may react either with neutral sterols or bile acids to produce weak carcinogens (75). Hill et al have pointed out that only four nuclear dehydrogenase reactions are sufficient to convert bile acids to a carcinogen and that surrogate reactions for these can be carried out on related steroids by lecithinase-negative clostridia isolated from feces (78). Clostridia capable of the reactions found by Hill's group have not been isolated in other laboratories (5). However, Hill's hypothesis suggests that high amounts of bile acids and the presence of clostridia with nuclear dehydrogenating activity in the feces are risk factors for colon cancer. Indeed it has been found that patients already

diagnosed to have colon cancer are more likely than control subjects to have one or both of these risk factors (79). The authors recognize that these results must be interpreted with caution since the characteristics of the feces in this experimental design may be a result of colon cancer rather than its cause.

Although colon cancer is relatively frequent in Western countries, even at its current incidence, it will be expected to affect only a relatively minor fraction of the population. Presumably the carcinogen involved will be a weak one, which by acting over a long period does not cause cancer until late in life. If there are compounds or bacteria in feces which may be related to this form of carcinogenesis, they may not be identified simply from random fecal samples. Their identification may require repeated measurements on individuals in a cohort large enough to yield a significant number of colon cancers. Hill and his co-workers have recognized this requirement and have designed a prospective study to test their hypothesis relating the risk of colon cancer to the amount of fecal bile acids and the presence of a particular clostridium.

A different chemical etiology for colon cancer is suggested by the isolation from extracts of human feces of a compound with mutagenic activity provisionally identified as an N-nitroso compound (80). N-Nitroso compounds are formed in a reaction between nitrite and either a secondary or a tertiary amine. The reaction can take place in an acid environment such as that in the stomach (81) or can be mediated by the anaerobic flora of the cecum (82). The nitrosatable amines required for the formation of N-nitroso compounds are present in the lower gastrointestinal tract as are the bacteria, but the source of nitrite has not been identified (83).

SUMMARY

Animal and bacteriological techniques have been developed for clarifying the role of the flora in the metabolism of drugs and other exogenous compounds. In general the flora tends to catalyze reductive and hydrolytic reactions, some of which reverse the detoxification reactions normally occurring in the liver. These reactions and others have been implicated in the pharmacological or toxicological action of exogenous compounds. Only in a few instances, however, have practical consequences of these reactions been documented. The major challenge at present is to develop methods capable of further defining the implications of reactions due to the flora.

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