Table 1. Conversion of *p,p'*-DDT to *p,p'*-DDD by coliform bacteria in broth culture; data from gas chromatography. Residues, after 24-hour incubation, from initial contents of 200 μg of *p,p'*-DDT.

Organism	Residue (μg)	
	p,p'-DDD	*p,p'*-DDT
E. coli	35.9	69.5
A. aerogenes	33.0	26.7
None	0	191.6

DDD, with at most a trace of *p,p'*-DDT. Essentially no chlorinated pesticides were found in the feces of the rats injected intraperitoneally; in fact the chromatograms did not differ significantly from those of extracts of the control feces.

Typical gas chromatograms of liver extracts are seen in Fig. 2. Analyses of livers from the animals to which *p,p'*-DDT was administered by stomach tube showed the presence of both *p,p'*-DDD and *p,p'*-DDT, with the ratio DDD : DDT varying from 1 : 3 to 1. On the other hand, livers from rats that received the *p,p'*-DDT intraperitoneally contained principally *p,p'*-DDT, the ratio DDD : DDT ranging from 1:24 to 1:60.

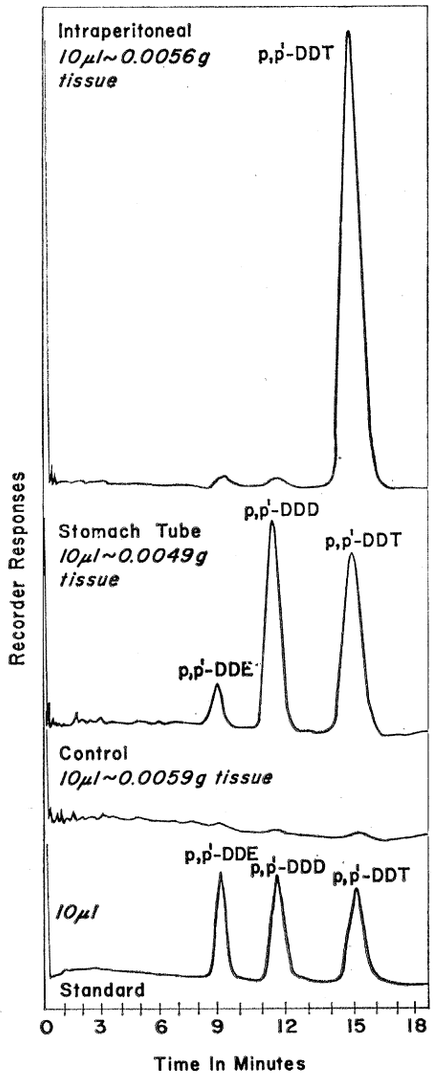

Fig. 2. Representative gas chromatograms of extracts of livers from the same animals and with the same standard as for Fig. 1.

The results of the analyses of feces and liver suggest that, if appreciable *p,p'*-DDD is to be found in the livers of animals administered *p,p'*-DDT, the *p,p'*-DDT must pass through the gastrointestinal tract. Because it has been demonstrated that one microorganism, yeast, can reductively dechlorinate *p,p'*-DDT to *p,p'*-DDD (*6*), and because the growth of Gram-negative rods in general is stimulated by *p,p'*-DDT (*7*), it seemed possible that coliforms—Gram-negative bacteria of the gastrointestinal tract—could be responsible for the conversion of *p,p'*-DDT to *p,p'*-DDD.

Representative studies show that the coliforms *A. aerogenes* and *E. coli* can effect the conversion (Table 1). In the bacterial cultures, *p,p'*-DDD constituted a significant fraction of the chlorinated pesticides recovered, with the DDD : DDT ratio ranging from about 1 to 1:2. Of the *p,p'*-DDT added to the cultures no more than 30 to 55 percent could be accounted for either as unchanged *p,p'*-DDT or as metabolite. However, incubation of uninoculated culture medium with *p,p'*-DDT resulted in neither appreciable loss of pesticide nor formation of *p,p'*-DDD. The loss of pesticide in the growing cultures may well represent the formation of other metabolites of *p,p'*-DDT that were not detected by the analytical methods employed.

Our results indicate that the normal flora of the gastrointestinal tract must be considered as the major agent for formation of *p,p'*-DDD in intact rats fed *p,p'*-DDT; they also indicate that the conversion takes place during the life of the animal, which fact does not support the suggestion of Barker *et al.* (*8*) that the DDD is a postmortem artifact caused by tissue decomposition and by invasion by adventitious microorganisms.

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8 November 1965

Autoradiography with Tritiated Methotrexate and the Cellular Distribution of Folate Reductase

Abstract. *Bound methotrexate has been revealed by an autoradiographic procedure, presumed to introduce a method for cytochemical study of folate reductase. Preferential localization is seen in kidney proximal tubules, intestinal epithelium, and nuclei of parenchymal liver cells in mice. The extremely firm binding and prolonged retention of this drug should render it suitable as an inert label for the autoradiographic study of cell migrations and lifetimes.*

The enzyme folate reductase (tetrahydrofolate dehydrogenase, *1*) catalyzes the conversion of folate and dihydrofolate to tetrahydrofolate. Several forms of this cofactor are required for many reactions that involve the transfer of one-carbon groups, and regeneration of tetrahydrofolate is essential for the synthesis of thymidylate (*2*). No cytochemical reaction has so far been devised for the demonstration of this important enzyme, nor, indeed, of any enzyme of this type. However, the intracellular sites of binding of suitable enzyme inhibitors can be revealed (*3*) by the use of an isotopic label (usually ^3H) in the inhibitor and the methods of tissue autoradiography. Using an irreversible inhibitor and strategies designed to confine it to known types of enzyme, Barnard and Ostrowski and their colleagues have shown that this approach can yield qualitative and quantitative information on the cellular distribution of cholinesterase and nonspecific esterases in various tissues (*3*). In the

case of folate reductase, a suitable enzyme inhibitor appears to be at hand, namely, methotrexate.

The antifolate drugs aminopterin and methotrexate (amethopterin) are retained in tissues for many months (*4*). The basis for this retention is a highly specific binding to folate reductase (*5, 6*). This binding is exceedingly firm in all mammalian sources studied, and a dissociation constant for the complex has been estimated (at *p*H 6) as about 3×10^{-11} *M* or less (*5, 7*). From quantitative studies on the loss of methotrexate from tissues of the mouse, it was concluded that this loss was due primarily to cell death and that it should be possible to use ^{3}H-methotrexate as a long-lived label for cells which were able to bind significant amounts of the drug (*8*). Indeed, the prolonged retention of ^{3}H-methotrexate has recently been employed in labeling granulocytes (studied in bulk, by liquid scintillation counting) by Bertino *et al.* (*9*). We describe here a procedure for autoradiographic localization of ^{3}H-methotrexate. It is presumed that the distribution observed is that of folate reductase in the respective tissues.

Young adult male Swiss mice were injected (0.1 mg of drug per kilogram of body weight) with ^{3}H-methotrexate (9.17 c/mmole; Radiochemical Centre, Amersham, England). One-half of the dose was injected by the intraperitoneal route and one-half subcutaneously. The mice were killed 24 hours later by decapitation. This dose was chosen as one which would result in near saturation of the folate reductase of liver and yet guarantee the absence of free drug in the tissues 24 hours after administration (*10*).

Weighed fragments of liver and kidney were taken from one such mouse, for estimation of both drug and enzyme content (Table 1), following procedures that have been fully described elsewhere (*5, 11*). From the same mouse, weighed fragments of liver and kidney were taken for measurements of radioactivity. Each fragment was frozen, thawed, minced, and then boiled for 5 minutes in a measured volume of distilled water, a procedure which liberates all the drug present into solution. An aliquot of each solution was taken after gently centrifuging down the cell debris, and its radioactivity was measured in a Packard Tri-Carb scintillation spectrometer. A dioxane-based scintillating fluid was used, and quenching corrections were made by means of external standardization. From the known specific activity of the methotrexate, its concentration was calculated (Table 1). Excellent agreement was found between these two methods of measurement. Furthermore, a small residual amount of free enzyme was detected. The data in Table 1, therefore, show that there is no free methotrexate present in the tissues actually used and that all the radioactivity recorded from them originates in bound methotrexate.

The autoradiography of liver, kidney, and duodenum was complicated by the solubility of the drug-enzyme complex. Folate reductase is known from biochemical studies (*5*) to occur in a soluble cell fraction, and in our studies evidence for its diffusibility was found in tissues processed by conventional methods for autoradiography. Modifications of the techniques described by Appleton (*12*) and by Miller, Stone, and Prescott (*13*) were therefore used. Immediately after the mice were killed, fragments of tissue no larger than 2 mm square were immersed in isopentane at the temperature of liquid nitrogen, transferred to liquid nitrogen, and then to a cryostat at −12°C, where sections were cut at 5 μm. In the Appleton technique, the sections were picked up from the cryostat knife, under a safelight, on cover slips coated with emulsion—either Kodak AR-10 stripping film or Ilford K2 liquid emulsion. The cover slips were at about −10°C at the moment of picking up the section and were immediately transferred to a container at −20°C for exposure. After exposure, which averaged 4 weeks, the cover slips were allowed to come to room temperature and were rapidly dried in a gentle current of air. This was sufficient to prevent loss of sections in subsequent processing. Fixation in 10-percent buffered formalin and washing in water preceded photographic development and fixation. In the alternative technique, sections were picked up from the cryostat knife on slides precooled to about −5°C and were dried (within 20 seconds) in a gentle current of air. They were then covered with a thin layer of Ilford K2 emulsion, prepared by dipping a stainless-steel loop in diluted, molten emulsion and allowing the film on the loop to dry for several minutes before applying it to the slide. Exposure again took place at −20°C, and formalin fixation preceded photographic processing. Both techniques gave consistent and reproducible results with tissues from six mice. The first (Appleton) method was judged superior for the purpose of observing intracellular distribution here. The resolving power of the autoradiographs (Fig. 1) was good, with little or no spread of radioactivity into tissue spaces around labeled cells.

Table 1. Bound methotrexate and free folate reductase in kidney and liver from one of the mice used for the preparation of autoradiographs.

Substance	Amount (in mμmole/g) in:	
	Kidney	Liver
Methotrexate:		
By ^{3}H measurement*	0.43	0.40
By enzyme assay†	.42	.36
Free folate reductase‡	.016	.008

* By scintillation counting, expressed as methotrexate equivalents. † Estimation by liberation of methotrexate and its titration against a standard enzyme solution (*11*). ‡ Estimation by assay of activity and calculation of amount of enzyme in methotrexate equivalents (*5*) based on the specific enzymic activity of folate reductase from tissues of control, noninjected mice. In view of the residual free enzyme in the tissues, it is clear that no free drug was present.

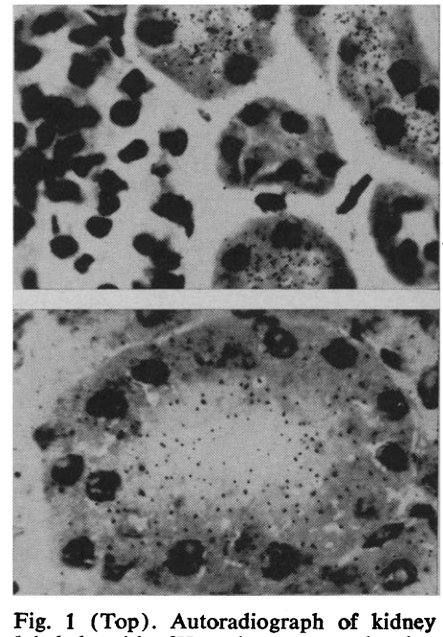

Fig. 1 (Top). Autoradiograph of kidney labeled with ^{3}H-methotrexate, showing silver grains distributed above proximal convoluted tubules (parts of three are seen in section), and showing the lack of label in the glomeruli (one seen on the left) and the very low level in the distal convoluted tubules (two are seen, center and lower right). Stained with hematoxylin only. (Bottom) A high-power view of a proximal convoluted tubule (from an autoradiograph similar to that at top). The cells are mainly labeled in the apical region.

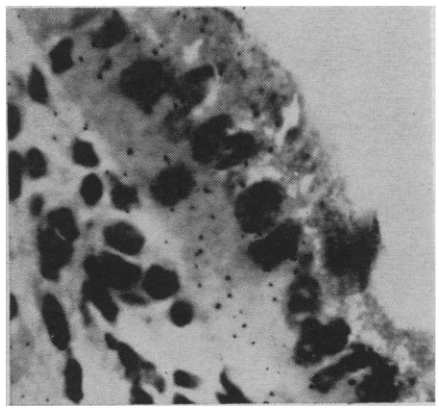

Fig. 2. Autoradiograph of intestine (parallel to autoradiographs of Fig. 1). The radioactivity is present in the epithelium of the villus, but at a lower concentration than in the proximal convoluted tubule of the kidney.

Tissues from control mice (injected with unlabeled methotrexate), processed similarly, showed no significant chemography.

In the liver, the parenchymal cells were uniformly labeled, whereas the Kupffer cells and connective tissues were unlabeled. Grain counts on AR-10 stripping film autoradiographs prepared by the Appleton technique were carried out on 300 randomly selected parenchymal cells: an area 6 × 6 μm over the nucleus was examined, and a similar area over the cytoplasm of the same cell. The mean grain count over the nucleus was 4.0 ± 0.12 (S.E.) grains per unit area; that over cytoplasm was 1.4 ± 0.07 grains per unit area. Background counts away from the tissue were below 0.1 grain per unit area. In the liver cells, therefore, the concentration of enzyme appears to be higher in the nucleus than in the cytoplasm.

In the kidney, the proximal convoluted tubules were heavily labeled: the grain densities over these cells were the highest seen in the tissues examined. No other structure in the kidney was significantly labeled (Fig. 1). In the proximal convoluted tubules, nuclei often appeared to be labeled, but the highest grain densities were seen over the cytoplasm of the apical half of the cell.

In the duodenum, labeling was limited to the epithelial cells (Fig. 2), which were radioactive both in the crypts of Lieberkuhn and on the villi. As in the kidney, nuclei sometimes appeared to be labeled, but the highest grain densities occurred over cytoplasm in the apical region of the cells.

It appears possible, then (on the basis of the evidence cited earlier for the specificity of methotrexate binding), to study the distribution of folate reductase in tissues by radioisotope cytochemistry and to compare its concentration in different sites on the basis of relative grain densities. The mere fact that localization of bound methotrexate can be revealed thus provides the basis of a new cell-tracing technique. This could be a valuable alternative to the use of ^{3}H-thymidine in studies of cell migrations and lifetimes.

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Antigenic Correspondence of Serum Albumins among the Primates

Abstract. *The relative cross-reactivities as judged by the quantitative precipitation reaction of 22 primate serum albumins were determined with pooled antiserums against human serum albumin. The antigenic correspondences to human serum albumin of all but three serum albumins fell into narrow ranges, and the groups defined by these ranges are distinct taxonomic categories. The values of the cross-reactivity ranges are consistent with the presumed phylogenetic relationships to man on the assumption that the evolutionary modification of protein structure has been progressive and divergent.*

Recent research correlating protein structure with systematics or phylogenetics has taken two directions. One is the analysis of primary protein structure (*1*, *2*). The other, immunochemical correspondence, while applied around the turn of the century by Nuttall (*3*), was refined and developed by Boyden and his colleagues (*4*, *5*). In recent years more strictly quantitative methods and more informative qualitative techniques have been used (*6–9*).

Our study was undertaken to provide and evaluate data obtained by quantitative precipitation on the cross-reactivity of antigens from a relatively large number of species in the same order of mammals. Serum albumins of primates were selected for several reasons. (i) Human serum albumin (HSA) is relatively well characterized immunochemically (*10*) and physicochemically (*11*). (ii) It is commercially available in a sufficiently pure state for immunization of rabbits. (iii) Serums from many living species of the Primates are obtainable. (iv) The understandable interest of biologists in the systematics and evolution of this order has provided information on the comparative anatomy of living and fossil forms, which is important for the interpretation of antigenic correspondence.

The order Primates can be subdivided into at least four major divergent groups with a considerable range in characteristics from primitive to modern (*12*). The Prosimii or "pre-monkeys" include the tree shrews, lemurs, pottos, and tarsiers; the Ceboidea are comprised of the New World or South American monkeys; the Cercopithecoidea or Old World monkeys include the African and