was added to the buffer at the time the gel was made and when the bridge solution was prepared; $FeSO_4$ (0.00001*M*) was also added to sharpen the transferrin zone. Electrophoresis was done in the vertical position for 6 to 7 hours and the voltage gradient set at 16 volt/cm across the gel.
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Chemical Carcinogenesis: Persistence of Bound Forms of 2-Fluorenylacetamide

Abstract. *The persistent binding of metabolites of hepatic carcinogen, 2-fluorenylacetamide, to glycogen and to DNA in a new population of liver cells, hyperplastic nodules, and to glycogen in liver cancer cells weeks to months after the carcinogen was removed from the animals' diet is indicated by spectrophotometric, chromatographic, and mass spectrographic data. This persistence of binding does not appear to occur in the nonhyperplastic or nonneoplastic liver surrounding the nodules or the cancer.*

For many different tissues, the required exposure time to a variety of carcinogens is considerably less than the shortest interval in which malignant cells can be detected with current techniques. Such observations have suggested that the rapid induction by cancer-inducing agents of irreversible tissue changes akin to mutations, which, by subsequent selection, lead to malignant neoplastic transformation.

In order to study the molecular pathology of liver carcinogenesis, we have fed rats a diet containing a carcinogen, 2-fluorenylacetamide (2-FAA), which induces a new population of liver cells, namely hyperplastic nodules (*1*). The hyperplastic nodules appear to be a site of origin for liver cancer and are: (i) quite uniform in morphologic and biochemical properties; (ii) large enough for gross identification and for isolation to enable appropriate biochemical, morphologic, and biological studies to be made. These two criteria are deemed essential for the analysis of the cellular and molecular events during carcinogenesis (*2*).

Male, white Wistar rats (Carworth Farms) (150 to 200 g) were fed the basal diet containing 2-FAA under conditions which lead to the induction of large hyperplastic nodules and hepatocellular carcinoma (*1*). Then, unless specified otherwise, all rats were fed the basal diet without the added 2-FAA for a minimum of 4 weeks before their being killed by guillotine. After decapitation, samples of hyperplastic nodules and carcinoma as well as of the nonhyperplastic or nonneoplastic liver surrounding these lesions were removed for histological study (*1*) and for extraction of glycogen (*3*) or DNA (*4*). Liver from control animals fed only the basal diet was examined similarly.

Purified glycogen (*3*) was extracted an additional three times with ethyl ether. After being dried, portions were dissolved in water and examined spectrophotometrically (Cary model 15 recording spectrophotometer) or hydrolyzed.

Glycogen from hyperplastic nodules, both before and after methanolysis or hydrolysis (see below), consistently showed a region of increased absorption between 265 and 305 nm, while glycogen from normal control liver or from the surrounding nonhyperplastic liver showed no absorption peak in this region (Fig. 1). Analysis of the small amount of glycogen from liver cancer many months after the inducing carcinogen 2-FAA had been removed from the diet showed an absorption spectrum similar to that from the nodule, whereas the glycogen from the surrounding nonneoplastic liver showed no such increased absorption (Fig. 1). Glycogen from control livers allowed to react with *N*-acetoxy-*N*-2-fluorenylacetamide (*N,O*-diacetyl-2-fluorenylhydroxylamine) (*5*) in vitro under N_2 at 37°C for 8 hours showed an absorption spectrum similar to that noted in either nodular or cancer glycogen.

Purified glycogen from hyperplastic nodules or from liver cancer was hydrolyzed for 20 hours at 70°C with 0.5*N* HCl in methanol or for 1 hour at 100°C in either 1.0*N* HCl or 1.0*N* H_2SO_4. This hydrolyzate, when separated with thin-layer chromatography (*6*), showed a minor component in addition to the major constituent, glucose. This extra spot had an R_F value different from that for glucose and was readily visualized as an area of fluorescence with a 254 nm lamp. Chromatograms of hydrolyzates of glycogen from the liver surrounding either hyperplastic nodules or carcinomas or of glycogen from liver of control animals did not have this component. The absorption spectrum of this extra component was similar to the spectra of *N*-acetoxy-2-FAA after it had been hydrolyzed and chromatographed in the same way as the glycogen from the nodule.

Polyacetate and trimethylsilyl esters of glycogen hydrolyzed by methanolysis were subjected to gas chromatography and mass spectrometry (*7*). In hydrolyzed glycogen from hyperplastic nodules and liver cancer, components which have retention times and mass numbers different from glucose were present. Some of those additional mass numbers suggest persistent binding of an aromatic compound to glucose within the glycogen.

Glycogen prepared from the livers of rats fed the diet with 2-FAA for 3 weeks, the basal diet for 1 week, and the carcinogen-containing diet for 2 additional weeks had an absorption spectrum very similar to that of glycogen from the hyperplastic nodules. Glycogen from animals fed this latter dietary regimen became radioactive when 9-^{14}C-*N*-hydroxy-2-FAA was fed or injected.

The above indicates that 2-FAA or its derivative [presumably esters of *N*-

hydroxy-2-FAA (*5*)] becomes bound to liver glycogen, and that a glycogen-bound form persists in the hyperplastic nodules and appears in the liver cancer long after the carcinogen has been removed from the diet. The chemically altered glycogen may be responsible, at least in part, for the lack of normal staining of glycogen as observed by electron microscopy and for some of the changes seen in glycogen metabolism in hyperplastic nodules (*1*).

Persistence of a bound metabolite of 2-FAA may also occur in the nodule in association with DNA. Purified DNA (*4*) from hyperplastic nodules but not from normal liver or liver surrounding the nodules had an absorption spectrum similar to that of normal rat liver or calf thymus DNA allowed to react in vitro with *N*-acetoxy-2-FAA (*5*). In addition, purified DNA from hyperplastic nodules contained a fraction with a bouyant density different from that of the DNA from liver of control animals or from nonhyperplastic liver surrounding the nodules. Most of the DNA from hyperplastic nodules was found to equilibrate in the gradient (*4*) in a position identical to that found for DNA from either control or adjacent nonnodular liver. However, about 1 to 2 percent of the DNA obtained from nodules equilibrated considerably below the major fraction.

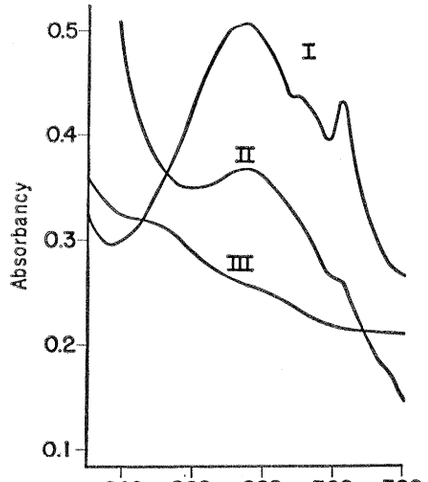

Fig. 1. Ultraviolet absorption spectra of hydrolyzed glycogen obtained from hyperplastic liver nodules induced by 2-FAA (*I*), from hepatocarcinomas induced by 2-FAA (*II*), and from nonhyperplastic liver adjacent to a 2-FAA-induced hyperplastic liver nodule (*III*). Preparations of hydrolyzed glycogen obtained from either liver adjacent to 2-FAA-induced hepatocarcinomas or control rat liver had a spectrum identical to *III*. These spectra were obtained with samples in methanol following hydrolysis for 20 hours at 70°C with 0.5*N* HCl in methanol. The hyperplastic nodule and adjacent liver glycogen were prepared from animals not exposed to 2-FAA in the diet for 4 weeks. The hepatocellular carcinoma glycogen was prepared from animals whose last exposure to 2-FAA was 6 months before they were killed.

Electron microscopic examination of DNA from normal liver, hyperplastic nodules, and surrounding nonhyperplastic liver by the Kleinschmidt technique showed that the nodule DNA could be regularly distinguished from the other two by excessive branching and frequent presence of strand separation (*8*).

It must be emphasized that careful histologic control is essential for the analysis of the liver surrounding hyperplastic nodules or cancer. The liver around the nodules may show cysts, cholangiofibrosis or other changes (*1*, *3*, *9*). Small hyperplastic nodules are easily overlooked grossly in this tissue.

Persistence of bound carcinogen after its removal from the diet may not be unique to 2-FAA, since radioactivity was found in liver DNA for 3 months after a single intraperitoneal injection of *p*-dimethylaminoazobenzene [butter yellow (*10*)]. In addition, alterations in glycogen have been reported in hyperplastic nodules induced by several carcinogens. (*1*). This may be a reflection of altered glycogen structure due to the presence of bound carcinogen.

Our data indicate (i) that persistence of a carcinogen bound to certain macromolecules, such as DNA, may be a prerequisite for carcinogenesis with some compounds. This would require reevaluating and possibly abandoning the "hit and run" or the "irreversible mutation" hypothesis (*9*). In viral carcinogenesis there is increasing indication for persistence of all or part of the genome of some oncogenic viruses (SV40, polyoma) throughout the whole process of neoplastic transformation (*11*). (ii) In view of the fact that the carcinogen or derivative is present in the original liver cells after 2-FAA is fed for a few weeks (*9*), in the cells of the hyperplastic nodule, and in liver cancer, but is not present in the liver surrounding the nodule or the cancer, there is established, for the first time, molecular evidence for the probable cellular lineage between the original liver and the cancer via the hyperplastic nodule. (iii) The presence of intact functioning and dividing cells in the nodule suggest that such biological behavior is possible when the cells contain some DNA which is obviously altered (for example, branching) by the probable presence of a metabolite of a carcinogen bound to it. (iv) The persistence of carcinogen bound to macromolecules, such as glycogen and DNA, and its apparent disappearance from such molecules in the surrounding liver suggest the possible importance of the absence of repair mechanisms as an essential step in carcinogenesis.

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