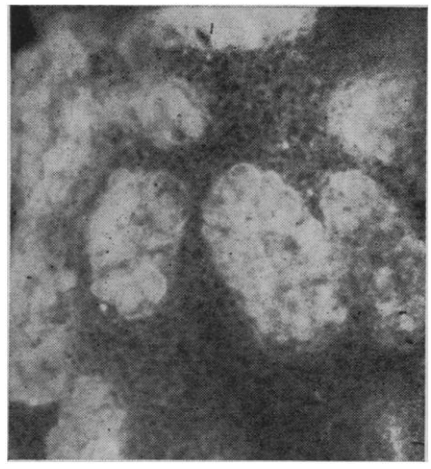

Fig. 3. Biopsy of rectal mucosa, from a patient with CFP, treated first with rabbit antiserum to CHO-I-C and then with fluorescein-labeled antiserum to rabbit γ-globulin; under the fluorescent microscope.

antiserum (*4*) against rabbit γ-globulin, antibodies were specifically absorbed onto these glandular structures (Fig. 3). Also, when the absorbed rabbit antiserum to CHO-I-C (which was specific for the abnormal unique glycoprotein) was conjugated with fluorescein (*5*) and used to stain the tissues, the results were the same. Appropriate control experiments confirmed the specificity of the reaction.

It may be argued that the immunologic techniques that we used are insufficiently sensitive to detect minor amounts of the unique antigen that may be present in CHO-I-N. Even if the antigen were present in an amount too small to give a positive reaction with the appropriate antibody, it might be capable of inducing formation of specific antibody when used for immunization. This possibility was tested: the precipitin produced by the unique antigen did not appear when CHO-I-C was allowed to react with an antiserum to CHO-I-N. Applications of other immunologic methods, such as hemagglutination and latex-particle agglutination, for identification of the antigen in CHO-I-N have failed thus far.

We considered the possibility that the absence of pancreatic enzymes might be responsible for the appearance of the mucopolysaccharide in CHO-I-C, because the unique antigen could not be demonstrated in the CHO-I-C prepared from stool of patients with CFP and receiving pancreatic enzyme (Cotazyme) orally. However, the antigen peculiar to cystic fibrosis of the pancreas could be detected under these conditions in the water-soluble fraction of the ethanol-benzene precipitate; this same fraction from normal subjects did not react with the antiserums against CHO-I-C to produce the unique precipitate, nor did the CHO-I prepared from stool of a depancreatized adult react in this way.

Our findings justify two conclusions: (i) absence of pancreatic enzymes is not responsible for the appearance of the unique mucopolysaccharide in the patients, and (ii) pancreatic enzymes influence the susceptibility of the unique material to denaturization, but do not alter its antigenic structure. When bile was used as source material for preparation of CHO-I-C the unique antigen appeared in the water-soluble fraction.

We have but limited knowledge of the antigen's chemical structure. Velocity centrifugation indicates a molecular weight exceeding 1 million. Strong-acid hydrolysis released reducing sugars (Somogyi-Nelson) (*6*) and also a substance that gives a color reaction, with Bial's orcinol (*7*) reagent, characteristic of pentoses. Treatment with a peptidase, Pronase (*8*), did not alter the specific antigenic structure although a considerable amount of amino acid was split off—as revealed by titration and amino acid analysis (*9*). The fact that the digested product gave a reaction of complete identity with the undigested material when tested by immunodiffusion does not exclude the possibility that amino acids are at least partly responsible for the specific antigenic structure; hydrolysis in strong acid, after Pronase treatment and subsequent dialysis, revealed a number of amino acids. The intrinsic structure of the mucopolysaccharide thus probably includes amino acids in addition to carbohydrates.

Our data agree with a hypothesis that the histologic pathology in cystic fibrosis of the pancreas reflects the elaboration of a mucus that is abnormal in structure and physical characteristics.

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Nucleic Acid Guanine: Reaction with the Carcinogen N-Acetoxy-2-Acetylaminofluorene

Abstract. *Reaction of* N-*acetoxy-2-acetylaminofluorene with DNA or RNA at* p*H 7 causes marked increase in absorption at 280 to 320 millimicrons and marked decrease in guanine content. Reaction with guanosine-8-*^{14}*C yields a radioactive fluorescent derivative. The data suggest that metabolic esters of* N-*hydroxy-2-acetylaminofluorene may be intermediates in the binding of this proximate carcinogen to nucleic acids in vivo.*

Studies in our laboratory have shown that the carcinogen 2-acetylaminofluorene (AAF) is metabolized by the rat and other species to *N*-hydroxy-AAF, which is more carcinogenic than the parent amide (*1*). Administration of 9-^{14}C-labeled AAF to rats results in low levels of DNA- and RNA-bound radioactivity in the liver; 2- to 3-times-higher levels are obtained when the *N*-hydroxy derivative is administered (*2, 3*). Identification of the metabolite(s), implicated in this reaction, with tissue nucleic acids is of particular importance in view of the possible causal relation between the reaction of carcinogens with DNA and RNA and the induction of neoplasia (reviewed by Miller and Miller, *4*).

Kriek (*5*) has shown that *N*-hydroxy-2-aminofluorene (*N*-hydroxy-AF) reacts with the guanine bases of RNA or DNA in vitro at *p*H lower than 6. This report shows that the guanine bases of the nucleic acids react readily at neutral *p*H with *N*-acetoxy-AAF,

whereas reactions with *N*-hydroxy-AAF and *N*-hydroxy-AF were not detected under these conditions.

Salmon-sperm DNA (Sigma) was dissolved (0.25 mg/ml) in 0.001*M* sodium citrate buffer, *p*H 8, denatured by heating at 100°C for 10 minutes, and then rapidly cooled. After titration to *p*H 7 with citric acid, 4.5-mg portions of the DNA were combined with 4.5 ml of ethanol, alone or containing 0.02 mmole of *N*-acetoxy-AAF (*6*) or other fluorene derivative, and incubated for 3.5 hours at 37°C. After extraction of the excess fluorene derivative with ethyl ether, the DNA was precipitated by addition of 2.5 volumes of ethanol and NaCl to a final concentration of 0.05*M* and dried under reduced pressure. The solution of the fluorene compounds, incubation, and extraction of excess fluorene derivatives were carried out in a nitrogen atmosphere to avoid oxidation of the fluorene compounds.

When the reisolated DNA was dissolved in 0.001*M* sodium citrate buffer, *p*H 7, the *N*-acetoxy-AAF–treated sample, as compared to the control DNA, showed a shift in the absorption maximum from 258 to 265 mμ and a pronounced shoulder at 300 to 315 mμ (Fig. 1*a*). Analysis of the bases by thin-layer chromatography (*7*), after hydrolysis of the DNA in formic acid in sealed tubes (*8*), revealed loss of most of the guanine: in a representative experiment it decreased from 26 mole percent in the original DNA to 4 mole percent in the DNA isolated after reaction with *N*-acetoxy-AAF. The other bases appeared unaffected by this treatment and, in terms of mole percentages, showed compensatory increases: 22 and 29 for adenine, 27 and 37 for cytosine, and 25 and 32 for thymine for the control and *N*-acetoxy-AAF–treated DNA hydrolysates, respectively.

A product insoluble in 1*N* HCl but soluble in ethanol was found in the formic acid hydrolysate from the *N*-acetoxy-AAF–treated DNA, but not in the hydrolysate of the control DNA; in ethanol it showed maxima at 282 and 332 mμ (Fig. 1*b*). Similar treatment of denatured DNA with *N*-hydroxy-AAF or *N*-hydroxy-AF caused no alteration in the spectral properties or base composition of DNA. *N*-Acetoxy-AAF reacted with native DNA to one-half the extent observed after denaturation.

Yeast soluble RNA (sRNA; Boehringer) reacted with *N*-acetoxy-AAF

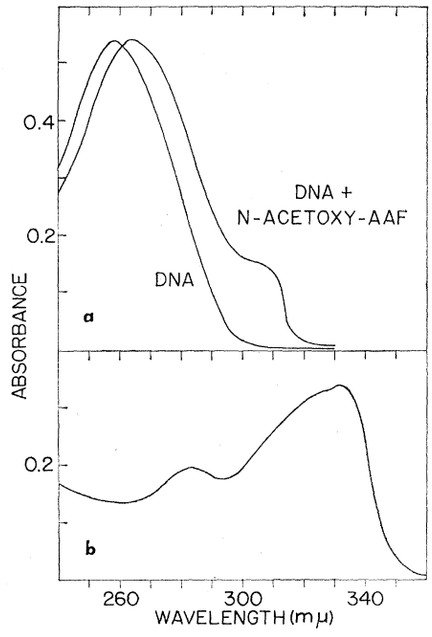

Fig. 1. Absorption spectra of: (*a*) denatured salmon-sperm DNA isolated after incubation at 37°C for 3.5 hours in 20 percent ethanol–0.001*M* sodium citrate buffer, *p*H 7, or in the same mixture containing 0.02 mmole of *N*-acetoxy-AAF per 4.5 mg of DNA; and (*b*) in ethanol, water-insoluble product obtained by formic acid hydrolysis of DNA or 1*N* HCl hydrolysis of guanosine that had been reacted with *N*-acetoxy-AAF at *p*H 7 and 37°C.

under conditions similar to those detailed above for DNA, or in a reaction system in which the amounts of *N*-acetoxy-AAF and of buffer were reduced to one-fourth of the levels employed with DNA. The sRNA isolated after reaction showed an alteration in absorption spectrum similar to that observed on reaction of DNA with *N*-acetoxy-AAF. The guanylic acid content of the sRNA, as determined by hydrolysis at 37°C in 0.3*N* KOH for 18 hours and ion-exchange chromatography (*9*), was reduced by one-third when 0.005 mmole of *N*-acetoxy-AAF was reacted with 4.5 mg of sRNA; the amounts of the other nucleotides were not decreased. Incubation of sRNA with *N*-hydroxy-AF, *N*-hydroxy-AAF, or 2-nitrosofluorene under these conditions caused no reduction in guanylic acid content.

Reaction also occurred on incubation at 37°C of 1 mg of guanosine with 0.5 to 4 mg of *N*-acetoxy-AAF in 2.5 ml of 20 percent ethanol–0.005*M* sodium citrate buffer, *p*H 7. Thin-layer chromatography of the ether-extracted reaction mixture on cellulose yielded a fluorescent spot with an R_F of 0.9 in a mixture of *n*-butanol, glacial acetic acid, and water at 40 : 10 : 50 by volume (*10*), or of 0.5 in *n*-butanol and water at 86 : 14 by volume with a beaker of 1*N* ammonia in the jar (*11*). The unreacted guanosine moved with R_F's of 0.3 and 0.0 in these solvent systems, respectively. When guanosine-8-^{14}C was included in the incubation mixture with *N*-acetoxy-AAF, 20 to 30 percent of the radioactivity was recovered in the guanosine; the remainder, in the fluorescent spot.

Hydrolysis of the reacted guanosine with 1*N* HCl for 1 hour at 100°C yielded an acid-insoluble, ethanol-soluble product having a spectrum identical with that of the insoluble product obtained by formic acid hydrolysis of the treated DNA (Fig. 1*b*); the spectrum, together with the ^{14}C content of the fluorescent product from guanosine-8-^{14}C, indicates that this substance contains both the purine base and the aminofluorene nucleus. Ribose and deoxyribose were presumably removed during the strong-acid hydrolyses and thus are probably not implicated in the reaction.

A fluorescent derivative was also obtained by two-dimensional thin-layer chromatography on cellulose of the nucleotides obtained by alkaline hydrolysis of sRNA treated with *N*-acetoxy-AAF. This fluorescent spot was not seen during chromatography of hydrolysates of untreated sRNA, and it separated from the normal nucleotides; it moved with an R_F of about 0.8 in a mixture of isobutyric acid, concentrated NH_4OH, and water at 66 : 2 : 32 by volume (see *12*), and with an R_F of 0.05 in 1*M* sodium acetate, saturated ammonium sulfate, and isopropanol at 18 : 80 : 2 by volume (*13*).

Several carcinogenic alkylating agents, and carcinogens metabolized to alkylating agents, have been shown to alkylate the 7-nitrogen atom of guanine in nucleic acids in vivo or in vitro or in both (*14*; reviewed in *4*). The nature of the reaction of guanine in nucleic acids and of guanosine with *N*-acetoxy-AAF is under investigation.

N-Acetoxy-AAF also reacts in vitro at neutrality with the sulfur atom of free and protein-bound methionine to yield sulfonium derivatives that break down at neutrality or (more rapidly) in the presence of alkali to yield 3-methylmercapto-AAF (*6*). *N*-Hydroxy-AAF and *N*-hydroxy-AF do not react with methionine under these conditions. 3-Methylmercapto-AAF is also

obtained by alkaline treatment of liver protein from rats administered AAF or *N*-hydroxy-AAF (*15*). Thus, although esters of *N*-hydroxy-AAF such as *N*-acetoxy-AAF have not yet been identified as metabolites, some reactive compound of this type (for example, *N*-acyloxy-AAF or the *N*-phosphate or *N*-sulfate of AAF) must be formed in vivo. The reactivity of *N*-acetoxy-AAF towards proteins and nucleic acids and the carcinogenicity of this ester for the subcutaneous tissue of the rat (*3*) suggest that metabolic esters of *N*-hydroxy-AAF may react with one or more critical cellular macromolecules to induce neoplasia.

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Diabetes, a New Mutation in the Mouse

Abstract. *Diabetes* (db), *which occurred in an inbred strain of mouse, is inherited as a unit autosomal recessive and is characterized by a metabolic disturbance resembling diabetes mellitus in man. Abnormal deposition of fat at 3 to 4 weeks of age is followed shortly by hyperglycemia, polyuria, and glycosuria. Accompanying morphological changes in the islets of Langerhans suggest neogenesis to compensate for insulin depletion.*

The mutation diabetes, which occurred in an inbred mouse strain (C57BL/Ks) at Jackson Laboratory, is characterized by a metabolic disturbance resembling diabetes mellitus in man. The diabetic mutant is similar to the obese mutant (*1*) in appearance but exhibits a severe disease syndrome with onset at an early age and a shortened life-span. Diabetes (*db*), like obese (*ob*), is inherited as a unit autosomal recessive with complete penetrance. Homozygotes are fat, hyperglycemic, and nonfertile; heterozygotes cannot be distinguished morphologically or physiologically from normal. Our preliminary observations on the inheritance, onset, and course of the diabetic condition and changes in the islets of Langerhans in a limited number of these mutants is reported. Other studies, including the chemistry, endocrinology, pathology, and genetics of the diabetes mutant, are in progress and will be reported in detail elsewhere.

Matings between mice heterozygous for diabetes and obese ($+db \times +ob$) resulted in 21 offspring, none of which exhibited the parental type of fat deposition. Therefore it was concluded that diabetes was not an allele of obese. Fifty-seven (30 percent) of 191 young born to $+db$ parents were of the diabetes (fat) phenotype and 13 (46 percent) of 28 offspring of $++$ by $+db$ parents were shown by progeny tests to be heterozygous for diabetes. Although *dbdb* females do not breed, their ovaries function normally when transplanted into a normal environment. Ten (43 percent) of 23 offspring resulting from crosses between females having *dbdb* ovaries and $+db$ males were of the diabetes phenotype. None of the above ratios is different from that expected of a unit recessive.

One distinguishing feature of the diabetes homozygote, deposition of fat in axillary and inguinal regions, is first noticeable at 3 to 4 weeks of age and, although not correlated with body weight, is unmistakable. Some homozygotes are small and plump and others are large and plump, the range in weights shown in Table 1 being considerable at all ages. Table 1 compares body weights, at weekly intervals, of diabetic (*dbdb*) and normal ($+db$ or $++$) mice and shows that in the former there is a rapid increase during the first few weeks with a plateau reached at about 10 weeks. One exceptional female attained a maximum weight of 62 g, whereas most females have not exceeded 55 g. This is in contrast to obese mutants (*obob*), in which the maximum weight attained is about 120 g. Not shown by figures in the table is the decrease in weight that occurs as the diabetics begin to succumb to the disease. This deterioration has occurred usually between 3 and 6 months of age, although two females survived 9 and 12 months. Obese usually survive about 15 months.

To determine the age of onset and degree of hyperglycemia, nonfasting blood sugar was determined weekly at the same time of day on groups of diabetic and normal mice of the same ages. For the assay, blood was withdrawn from the orbital sinus into a 50-μl pipette, deproteinized in 5 ml of tungstic acid reagent, and assayed for total reducing sugars by the micromethod of Folin and Malmros (*2*).

Results of the assays are given in Table 2. The blood sugar of control mice did not exceed 200 mg/100 ml of blood at any age, whereas all diabetics had concentrations of at least 200 mg/100 ml by 8 weeks of age, and all reached or exceeded a level of 300 mg/100 ml by 10 weeks of age. Although 28 percent of diabetics had blood sugar concentrations of 200 mg/100 ml when 3 to 4 weeks old, others did not reach this level until they were 8 weeks old. Our records suggest that the latter were more often females than males, and also that males tended to reach blood sugar concentrations of 500 mg/100 ml at earlier ages