Reports

Blocked Kynurenine Pathway of Tryptophan Metabolism in Hepatoma

Tryptophan metabolism is related to nicotinic acid which, like several of the B-complex vitamins, is reduced in tumor tissues (*1*). The concentration of coenzyme I (CoI) and coenzyme II (CoII) is relatively low in experimental and human tumors (*2, 3*). The concentration of coenzyme I decreases continuously in the liver of rats that are fed with *p*-dimethylaminoazobenzene (*3*). An increase in the reduced form over the oxidized form of coenzyme I was observed in Jensen sarcoma (*4*). The urinary kynurenine excretion was found to be high in cancer patients on high tryptophan diet (*5*). These observations suggest a disturbance in the tryptophan metabolism.

Rats of the Fisher strain (*6*) and C3H No. 129 mice (*7*), bearing transplanted hepatomas, were used in these investigations (*8, 9*). Homogenates of normal liver, host liver, and tumor were used to determine the tryptophan peroxidase (TP) activity and kynurenine degradation. Rat tumors were large, nonulcerated, containing necrotized parts, and the mice tumors were medium size. Only the nonnecrotized part of tumors was used. The homogenate was strained through cheesecloth before use. Knox and Mehler's method (*10*) was used to determine tryptophan peroxidase activity.

Because the kynureninase spectrophotometric method (*10*, p. 242) was found unsatisfactory for our purpose, the following procedure was used in determining kynurenine degradation. Fresh tissue was homogenized with cold 0.067*M* phosphate buffer at *p*H 7.5 (1 g of tissue/2 ml of buffer); 0.2 ml of homogenate was added with 0.4 ml of buffer, 0.2 ml of pyridoxal phosphate (0.1 mg/ml), and 0.2 ml of 0.02*M* kynurenine. The tissue blanks consisted of 0.2 ml of additional buffer instead of kynurenine; the control had 0.2 ml of additional buffer instead of tissue homogenate.

Two sets of tubes were prepared for each type of mixture. One set was inactivated after 5 minutes by adding 2.5 ml of a mixture of ethanol and acetic acid, (9 parts of alcohol/1 part of glacial acetic acid). The second set was incubated for 2 hours under N_2 at 37°C, then inactivated in the same way as the first set.

The deproteinized, incubated and nonincubated samples were centrifuged, and 2 ml of the supernatant was evaporated on a steam bath. The residue was dissolved in 1 ml of 95-percent alcohol.

The mixture of alcohol and residue was centrifuged, and 0.25 ml of the supernatant was spotted over a quarter-inch area, using Whatman No. 1 chromatographic strips, 1 by 20 in. Evaporation of the sample was hastened by means of infrared heat over an atmosphere of nitrogen.

The strips, attached to glass rods with nonstaple paper fasteners, were developed by descending chromatography for 24 hours in a Chromatocab (*11*) (Fig. 1). The solvent used was water-saturated butanol. After they had been dried in a fume hood, the chromatograms were sprayed with 0.25-percent ninhydrin in water-saturated butanol and heated in an oven for 15 minutes at 75°C.

The mean value of tryptophan peroxidase activity, expressed in micromoles of kynurenine per gram of dry tissue, per hour, was found to be 2.00 for normal rat liver, 3.91 for rat host liver, and 0.29 for the rat hepatoma. In the mouse, the figures were 5.20 for normal liver, 3.11 for host liver, and 0.00 for the hepatoma. In normal liver, the added kynurenine disappears before incubation (chromatogram No. 4).

The ninhydrin-reacting substances formed through degradation of kynurenine appear only after incubation (chromatogram No. 5).

Immediate disappearance of kynurenine may be explained by combination with pyridoxal phosphate under the catalytic action of normal liver homogenate. This eventual combination is absent in tumor homogenates (chromatogram No. 8). The enzyme responsible for the disappearance of kynurenine in normal liver homogenate is under investigation.

It seems that, during incubation, substances that react with ninhydrin are produced from kynurenine as it disappears. This reaction results in further transformation of ninhydrin-colorless compounds, probably by transamination with keto acids that are present or are formed during incubation; it also results in alanine formation (kynureninase action). Wiss reported kynurenine transamination (*12*) and alanine formation (*13*).

Only a small amount of ninhydrin-colored products is produced after incubation of normal liver in the absence of kynurenine (chromatogram No. 3). Neither ninhydrin-colorless nor ninhydrin-colored products are produced in

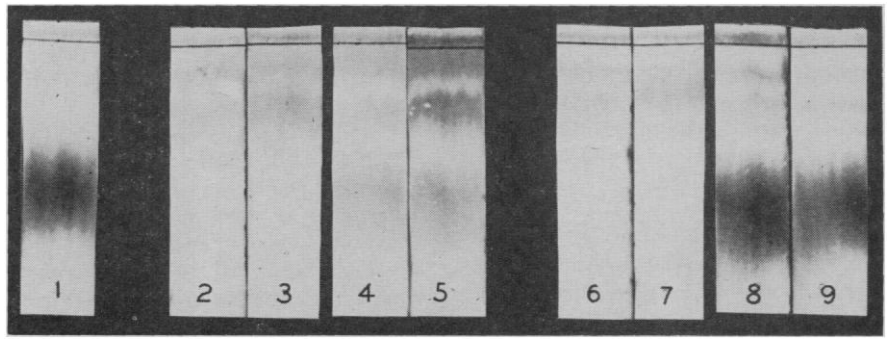

Fig. 1. Anaerobic kynurenine degradation in normal liver and transplanted hepatoma of the rat. 1, Kynurenine control without homogenate, incubated; 2, normal liver control, nonincubated; 3, normal liver control, incubated; 4, normal liver added with kynurenine, nonincubated; 5, normal liver added with kynurenine, incubated; 6, hepatoma control, nonincubated; 7, hepatoma control, incubated; 8, hepatoma added with kynurenine, nonincubated; 9. hepatoma added with kynurenine, incubated. All samples were added with pyridoxal phosphate. The incubation time was 2 hours under N_2. The nonincubated samples were inactivated 5 minutes after kynurenine was added to the homogenate mixtures.

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appreciable amount in incubated tumor homogenate, whether or not the homogenate is added to kynurenine (chromatograms No. 7 and No. 9).

The blockage of the ring opening and the blockage of the steps following the kynurenine pathway of tryptophan metabolism, reported here, indicate, in tumors, a marked decrease of tryptophan metabolism via kynurenine. A similar blockage of the ring opening was observed for histidine in hepatoma (*14*).

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Eastern Equine Encephalomyelitis Virus Isolated from Three Species of Diptera from Georgia

The virus of eastern equine encephalomyelitis has been isolated from Diptera four times since Kelser demonstrated that *Aedes aegypti* could transmit the virus in 1933. The significance of these isolations has been obscured by the difficulty in demonstrating experimental transmission with the species involved (*1*). Few would consider a species of insect a natural vector of a virus unless the virus could be isolated from it during an epizootic and unless the virus could also be transmitted experimentally by it.

The virus of eastern equine encephalomyelitis was isolated from pools of three dipterans: *Aedes mitchellae* (Dyar), *Anopheles crucians* Wiedemann, and an unknown species of *Culicoides* that were captured in southern Georgia in July 1956 (*2*). The isolation from *Aedes mitchellae* is probably most significant for species of this genus of mosquitoes have been effective experimental vectors of the virus (*3*). Chamberlain *et al.* (*4*) determined the threshold of infection, the transmission rate, and the infection rate of 20 species of mosquitoes. Excellent vector potentials were shown by three species, all belonging to the genus *Aedes*. *Anopheles crucians* was rated poor. *Culicoides* were not tested.

The mosquitoes and midges were collected in modified New Jersey type light traps on farms where horses had eastern equine encephalomyelitis (*5*). The live insects were anesthetized by chloroform and identified as to species in the case of mosquitoes and to genus in the case of *Culicoides*. Pools of one to 20 individuals were immediately ground in Ten Broeck tissue grinders with 1 ml of sterile, distilled water containing 5000 international units of penicillin with 5 mg of streptomycin. The resultant suspensions were centrifuged, and the supernatant fluids were inoculated into the allantoic chamber of 8-day embryonated chicken eggs, six eggs usually being used per inoculum, and each egg receiving 0.1 ml of fluid (*6*). Following inoculation, the eggs were incubated at a temperature of 35°C for 8 days. Allantoic and amniotic fluids were harvested from all embryos that died within this period and tested for bacterial contamination on nutrient agar and in thioglycollate broth. Harvests that appeared to be bacteriologically sterile were inoculated in further series of eggs. Isolates were sent to our laboratory in Wisconsin, where they were identified by titration with and without specific antiserum.

The pool of *Aedes mitchellae* from which virus was isolated was collected on 28 July from a farm near the town of Patterson in Pierce County. Eastern equine encephalomyelitis virus was isolated from the brains of two horses on this farm. The pool of *Anopheles cricians* was collected 30 July, on a farm in Appling County. The *Culicoides* from which the isolation was made were collected 28 July on a farm in Wayne County. Virus was recovered from a horse on this farm. The isolation history is given in Table 1.

The initial inoculum of the infected insect tissue killed half or more of the embryos after an unusually long incubation period of 69 to 144 hours. The period was reduced in the second or third passages to the characteristic time of 18 to 23 hours. The culture from *Anopheles* took four passages before all embryos were killed, and during this period the virus appeared to be sensitive to the effect of dilution and to freezing and thawing. Virus diluted in broth alone had a titer of $10^{3.5}$ as compared with its titer of $10^{5.8}$ when it was diluted in normal serum and broth. The adapted virus of the third or fourth passage of all three isolates possessed an embryo lethal titer of $10^{5.2}$ to $10^{6.2}$. Specific eastern equine encephalomyelitis antiserums prepared in chickens neutralized $10^{1.7}$ to $10^{2.8}$ LD_{50} of virus. Normal chicken serums and western equine encephalomyelitis antiserums did not neutralize the isolates.

All three dipterans from which the isolations were made are common in Georgia but are not widely distributed in other parts of the United States (*7*). *Aedes mitchellae* seems to be limited largely to the Atlantic and Gulf coastal plains, but, unlike its salt-marsh relative *Aedes sollicitans*, it breeds in fresh-water pools. Adults and larvae are seen throughout the year in southern Georgia. The range of *Anopheles crucians* is similar to that of *Aedes mitchellae*, but it extends further north and south, having been reported from Massachusetts and Central America. Its greatest abundance is reached in the cypress swamps of Georgia and Florida, where the larvae thrive in the acid waters of the swamps. Female *Anopheles crucians* are indistinguishable from *A. bradleyi* and *A. georgianus*. *Culicoides* are prevalent along the eastern seaboard in the tidewater counties where outbreaks of eastern equine encephalomyelitis have occurred most frequently.

Although nothing has been published about *Culicoides* and equine encephalomyelitis virus, the vector efficiency of the genus has been demonstrated for the blue tongue virus of sheep, and *Culicoides* are reported to transmit African horse sickness and fowl pox viruses (*8*). Robert Levi-Castillo of the Public

Table 1. Isolation and identification.

Source	Passage history									
	First		Second		Third		Titration	Neutralization		
	Mortality (No.)	Incubation (hr)	Mortality (No.)	Incubation (hr)	Mortality (No.)	Incubation (hr)		EEE	WEE	N
Aedes mitchellae	3/6	144–180	2/3	40–72	3/3	18–22	5.2	1.7	0	0
Culicoides spp.	4/6	136–180	2/3	23			6.2	2.7		
Anopheles crucians	3/3	69–144	4/20	24–96	1/10	24	5.8*	2.8	0	*

* Titer with normal serum; see text.