TABLE II.
Liver Protein, Pseudocholinesterase Activity of the Plasma and Hepatoma Incidence in Rats Fed 2-acetylaminofluorene and in Pair-fed Controls. Four animals in each group—a control group and a drug group sacrificed at the end of each period.

Wks	Liver protein conc. g protein/100 g liver		Pseudocholinesterase activity μl CO_2/ml/hr		*"t"	*"P"	Hepatomas, %	
	Controls	Drug†	Controls	Drug†			Controls	Drug†
8	18.7	17.5	92	193	7.9	<.01	0	0
14	16.7	17.4	71	169	3.3	.015	0	50
24	19.2	15.9	68	160	7.4	<.01	0	75
32	18.7	12.4	86	110	2.5	.04	0	100

* "t" and "P" values refer to the relationship of the pseudocholinesterase values obtained for the drug-fed animals to those obtained for the control animals for each period.
† Drug withdrawn after 14 wks.

other hand rose to a level approximating twice that of control values. After 24 weeks, at which time the drug-fed animals displayed the initial evidence of decrease in liver protein concentration, pseudocholinesterase activity values were still significantly higher than control values. After 32 weeks, when liver protein concentration was quite low in the animals fed 2-acetylaminofluorene, plasma enzyme activity remained slightly higher than corresponding control levels. It should be noted however, that the total liver protein increased in the drug-fed animals as a result of an increase in size of the liver. This increase in size was associated with abnormally high water and fat concentrations but with an abnormally low protein concentration.

The mechanisms by which plasma pseudocholinesterase is stimulated by the feeding of 2-acetylaminofluorene remains obscure at this time. Attention is drawn, however, to the fact that the rise in plasma pseudocholinesterase activity precedes any gross evidence of hepatoma, and that the activity of the enzyme does decrease significantly following this rise,—a decrease which may be associated with depletion. These observations are receiving further study.

Summary. Evidence is presented for the identity of the plasma pseudocholinesterase of dog and rat and that the latter enzyme is stable when stored at low temperatures. Protein starvation is accompanied by a decrease in rat plasma pseudocholinesterase activity. The activity of this enzyme has been shown to increase markedly in rats fed 2-acetylaminofluorene before any gross evidence of hepatoma can be detected. As the hepatoma develops and liver protein concentration declines, the pseudocholinesterase activity of the plasma decreases from this high level.

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Methylene Blue Potentiation of Alloxan Diabetes.* (17670)

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Highet and West(1) reported that the intraperitoneal injection of methylene blue (1 cc of a 1% solution per rat), administered 30 minutes prior to alloxan protected

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1. Highet, Doris H., and West, Edward S., *J. Biol. Chem.*, 1949, v178, 521.

TABLE I.
The methylene blue was injected intravenously into the tail vein in doses of 35-50 mg/K (3.5-5.0 cc of 1% MB/K) immediately preceding the alloxan (30-40 mg/K).

No. of animals	Avg initial wt	Alloxan dose, mg/K	Methylene blue dose, mg/K	% diabetic	P value	Avg 48-hr blood sugar	
						Diabetic	Non-diabetic
6	149	40	0	100		389	—
3	156	40	50	100		500	—
44	142	30	0	52		338	119
17	136	30	35	100	<.001	420	—

rats against diabetes. The alloxan was also injected intraperitoneally, in doses of 150 mg/K. Since we previously found that the production of diabetes by intraperitoneal injection of alloxan was subject to great variation, as compared to the diabetes produced by its intravenous injection, and since this mode of administration required about 5 times the dose of alloxan(2), it was thought desirable to repeat the methylene blue experiments using the intravenous route.

Methods. Sprague Dawley rats were used in this study. They were allowed food and water *ad lib.* The alloxan monohydrate solutions were prepared in concentrations of 1.5 and 2.0% and they were injected intravenously (0.2 cc per 100 g of body weight) in doses of 30 and 40 mg/K. The technique of intravenous injection and blood sampling was carried out in the manner previously described(2). Methylene blue was prepared as a 1% aqueous solution and was injected intravenously in doses ranging from 3.5 to 5.0 cc/K (35-50 mg/K). Since the dose of methylene blue used by Highet and West was 1 cc of 1% methylene blue per rat (rats weighing between 189 and 310 g) this dose was equivalent to 32-53 mg/K. Blood samples were drawn for sugar determinations by the Folin Malmros micro method(3) at 24, 48, and 72 hours after the injection of the alloxan. The rats were considered diabetic if any of the blood sugar values were greater than 150 mg %. As a rule the 48 hour blood sugar values were in the neighborhood of 300 to 400 mg %.

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3. Folin, O., and Malmros, H., *J. Biol. Chem.*, 1929, v83, 115.

Experimental. Potentiating action of methylene blue on alloxan diabetes. The effect of methylene blue injection on the production of alloxan diabetes is shown in Table I. It was previously established(2) that when 40 mg/K of alloxan were injected into fed rats, 95% were rendered diabetic. The 6 rats injected with this dose agreed with this. However, only 3 of the 6 rats which received methylene blue intravenously (50 mg/K) immediately before being given this alloxan dose survived and all of these developed diabetes. Their average 48 hour blood sugar value was higher (500 mg%) than was the case in the rats receiving only alloxan (389 mg %).

When the intravenous alloxan dose was reduced to 30 mg/K, diabetes was produced in only 52% of the 44 rats injected. When methylene blue was injected in doses of 35 mg/K immediately preceding this dose of alloxan all of the rats developed diabetes and again the average 48 hour blood sugar value was higher.

These findings disagree with those reported by Highet and West. The intravenous injection of methylene blue immediately preceding the alloxan, sensitized the rats to the development of alloxan diabetes. Both the incidence and severity of the diabetes was increased. Statistical analysis of the data indicates that the potentiating action of methylene blue is highly significant ($P < .001$).

Since we injected the methylene blue immediately preceding the alloxan, whereas Highet and West injected the methylene blue 30 minutes before, it seemed possible that their reported protection might be due to a delayed effect which appeared after a latent period. Eight rats were therefore injected

with methylene blue (35 mg/K) and after 60 minutes were given a diabetogenic dose of alloxan (40 mg/K i.v.). However, as in the previous experiments, no significant protection was observed.

Reactions of methylene blue with glutathione and dialuric acid, and the mechanism of methylene blue potentiation. It has been reported that methylene blue is reduced by glutathione and cysteine(4). Since glutathione injection protects rats against the development of alloxan diabetes(5,6), any factor which lowers the concentration of reduced glutathione should sensitize animals to the development of alloxan diabetes(7). However, in preliminary studies carried out *in vitro* under anaerobic conditions at pH 7.4, glutathione, in concentrations of 600 mg/L, (which is equivalent to a blood GSH of 60 mg/100 cc) was found to reduce methylene blue (35 mg/L) only slightly in 30 minutes.

When alloxan is injected into an animal it is destroyed by several reactions, one of which is its reduction to dialuric acid. This can be accomplished by cysteine(8,9,10), glutathione(9,10), and the reduced coenzyme DPN-H_2(11). It now seems fairly well established that when alloxan is reduced to dialuric acid it loses its diabetogenic potency(9). Cysteine, which rapidly accomplishes this reduction *in vitro*(8,12), also protects rats against alloxan diabetes(6). It would also be expected that any compound which is capable of reoxidizing the dialuric acid thus formed back to alloxan, might potentiate the production of diabetes. We have found in preliminary studies that visible reduction of methylene blue occurs within 5 minutes when dialuric acid (40 mg/L) was added to a methylene blue solution (35 mg/L) anaerobically at pH 7.4. It may be presumed that the methylene blue oxidizes the dialuric acid to alloxan.

Discussion. Although we are unable to account for the reported findings of Highet and West, the following may possibly explain their results: (1) Diabetes may have been produced, but it may have been missed because of a delayed hypoglycemic or norglycemic phase which occasionally occurs as late as 48 or 72 hours(2). (2) The intraperitoneal injection of near toxic doses of methylene blue modified the physiological state of the peritoneal cavity and interfered with the subsequent absorption of alloxan. Since alloxan is rapidly decomposed to a nondiabetogenic compound at pH 7.4(9,12), such a delay in its absorption would be equivalent to decreasing the injected dose of alloxan.

The possibility that alloxan or dialuric acid may occur within the human body has been considered(7). The potentiating action of methylene blue serves to emphasize that potential diabetogenic compounds which might normally be present within the human body could be converted to active diabetogenic compounds under appropriate conditions. Recently Patterson has shown that large doses of the oxidized form of vitamin C, *i.e.* dehydroascorbic acid will produce a permanent diabetes in rats(13). Incidentally, both dialuric acid(11) and ascorbic acid(14) are oxidized to their respective diabetogenic compounds by cytochrome *c*. Therefore the possibility that abnormal metabolic conditions may favor the appearances of diabetogenic compounds within the human body needs careful investigation.

Summary. 1. Methylene blue injected intravenously in doses of 35 mg/K immediately preceding alloxan (30 mg/K i.v.) increased

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13. Patterson, J. W., *J. Biol. Chem.*, 1950, in press.
14. Szent Gyorgyi, A., *Biochem. J.*, 1928, v22, 1387.

the incidence of diabetes from 52 to 100%.

2. The potentiating effect of methylene blue is attributed, in part at least, to its ability to reoxidize dialuric acid back to alloxan and thereby in effect it is equivalent to increasing the effective dose of alloxan.

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Absorption, Excretion, and Toxicity of Milibis* (Bismuthoxy-p-N-glycolyl-arsanilate) Following Oral Administration. (17671)

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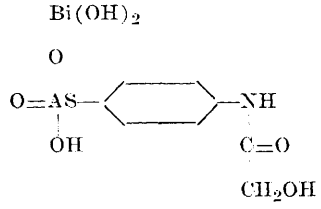
Milibis, a drug which promises to become important in the treatment of amebiasis, is a salt-like complex derived from bismuth trioxide and p-N-glycolyarsanilic acid. It has the approximate composition $C_8H_9AsBiNO_6 \cdot xH_2O$, and was first studied by Hauer(1) under the name of "Wia". The probable structure of its principal component, bismuthoxy-p-N-glycolylarsanilate, is as follows:



In the product as supplied, a small amount of the di-substituted compound $(C_8H_9AsNO_5)_2BiOH$, is probably also present. Milibis has been found quite effective as an amebicide in both experimental animals(2,3) and human subjects(1,2,4-6) as well as for the treatment of experimental yaws and syphilis in rabbits(2). From the very low aqueous solubility of the product† it could be predicted that only small amounts would be absorbed following oral administration, but obviously the product must dissolve to some extent if microorganisms are to be attacked. In view of the toxic potentialities of As and Bi compounds, it has been of interest to determine specifically the amounts of these elements absorbed following oral administration of the drug, and to ascertain whether appreciable quantities tend to accumulate in the tissues.

Acute toxicity. No toxic manifestations were apparent following single oral doses of 2.5, 5, or 10 g/kg, in either mice or rats. Groups of 3 rats, and 9 mice, were used at each dose level.

Subacute toxicity. Four groups of 5 rats weighing initially 130-140 g were given single oral doses of 2.5, 5, 7.5, and 10 g/kg by stomach tube, on 6 days of each week for 3 weeks. The drug was administered as a suspension in 0.5% aqueous gum tragacanth, and in a volume of 2 cc/100 g. Food (Purina Dog Checkers) and water were provided *ad lib.* The rats were weighed frequently so that comparisons could be made with a control group of 5 animals. One rat in the 5 g/kg and 2 rats in the 10 g/kg group did not survive the experiment. Two of these deaths were obviously accidental, due to difficulties in ad-

* Registered trademark of Winthrop-Stearns Inc.

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† The solubility of Milibis in water, ethanol, acetone, and propylene glycol is less than 0.025% at room temperature.

Some specific solubilities (per 100 cc of solvent) are as follows: 20% ethanol, 0.2 mg; 30, 50, and 95% ethanol, each 0.3 mg; 0.1 N HCl, 1.5 mg. These figures were obtained by determining the Bi content of a solution which had been saturated at room temperature, and calculating the Milibis content therefrom.