chloric acid for 1 hour at 100°C resulted in its complete conversion to azo-pigment A. For each micromole of hydrolyzed azo-pigment B, 1 μmole of glucuronic acid was liberated, as determined by the carbazole method (*8*). Data of a typical experiment are given in Table 1. Hydrolysis could also be achieved by incubating azo-pigment B with β-glucuronidase of animal or bacterial origin (Table 1).

Since fresh bile and urine from jaundiced patients yielded almost exclusively azo-pigment B, and since 2 moles of hydroxypyrromethene diazonium salt are produced from 1 mole of bilirubin (*6*), it would appear that direct-reacting bilirubin is conjugated with 2 moles of glucuronic acid. In analogy with other instances of glucuronide formation, it may be assumed that the glycosidic linkage occurs at the α,α′-hydroxy groups of bilirubin. The finding in bile and urine of minute amounts of material giving rise to azo-pigment A together with azo-pigment B suggests that small amounts of direct-reacting bilirubin may be present as a monoglucuronide. This observation is in agreement with an earlier report, indicating the separation of two closely related water-soluble fractions of bilirubin from bile (*9*). While this work was in progress, Billing and Lathe published an abstract in which they also suggest that, in bile, bilirubin is excreted as an "ester glucuronide" (*10*).

In the serum of patients with regurgitation jaundice, conjugated bilirubin was found to predominate (direct-reacting bilirubin). On the other hand, in retention jaundice, most of the serum pigment was shown to be free bilirubin (indirect-reacting bilirubin). Since, in jaundiced patients with bilirubinuria, the urinary bilirubin was found to yield almost exclusively azo-pigment B, it appears that the kidneys can excrete only conjugated bilirubin. In a child with congenital nonhemolytic jaundice, exhibiting 30 mg percent of free bilirubin in the serum (*11*), no bilirubin could be found in the urine (*12*).

These findings demonstrate that, in the serum, glucuronic acid-conjugated bilirubin gives the direct van den Bergh reaction, whereas free bilirubin, owing to its insolubility in water, requires the prior addition of alcohol to initiate the coupling with the diazo reagent. In bile, most or all of the bilirubin is excreted as a water-soluble glucuronide. In regurgitation jaundice, conjugated bilirubin gains access to the blood and hence to the urine, resulting in bilirubinuria.

RUDI SCHMID, M.D.
National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, U.S. Public Health Service, Bethesda, Maryland

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Solute and Temperature Effects in the Pervaporation of Aqueous Alcoholic Solutions

The phenomenon of pervaporation was first observed by Kober (*1*), who originated the term. It may be defined as the passage of a liquid through a semipermeable membrane and subsequent evaporation of the liquid. Farber (*2*) pointed out the usefulness of pervaporation in concentrating protein solutions. More recently, pervaporation and related phenomena have been discussed by da Fonseca (*3*).

Although the few available references cite the advantages of using pervaporation in certain chemical and biological researches, this technique has not been used to any great extent. In connection with food studies, we have used pervaporation to dehydrate mashed potatoes. Our methods may also be applicable in phases of other research—for example, in dehydrating plant tissue or in removing water from aqueous alcoholic extracts.

When a solution of equal parts by weight of ethanol and water was placed in a cellophane bag that was suspended in a forced-draft oven maintained at 60°C, the water content of the aqueous alcohol that passed through the membrane was about 65 percent. (The bags were formed from seamless, regenerated cellulose that is manufactured specifically for dialysis. Although wall thickness was not critical, a 0.0016-in. membrane was ordinarily used.) However, when a suspension of mashed potatoes in 1/1 ethanol-water was treated in the same manner, the water content of the pervaporated vapor was about 95 percent. Essentially the same result was obtained when the filtrate from a mixture of mashed potato, ethanol, and water was pervaporated, indicating that the higher ratio of water to alcohol must be due to the presence of the soluble potato solids.

Soluble potato solids consist principally of sugars, citric and other organic acids, nitrogen compounds, and inorganic salts. The naturally occurring mixture of water-soluble potato solids is also soluble in 1/1 ethanol-water but is only slightly soluble in ethanol.

To study the effect of the presence of a solute on the pervaporation of an aqueous ethanol solution, 1/1 ethanol-water mixtures containing various solutes (2.5 percent added) were pervaporated. Some of these solutes (class I) are soluble in ethanol and represent various degrees of solubility in water. Other solutes (class II) are soluble in water and are only slightly soluble in ethanol. The solubilities of the various solutes in water and in alcohol and the percentages of water in the transmitted vapor are listed in Table 1.

Our class-I solutes included citric acid, hydroquinone, and benzoic acid; although citric acid is quite soluble in water, hydroquinone is moderately soluble, and benzoic acid is only slightly soluble. In pervaporating solutions containing class-I solutes, the percentage of water in the pervaporated vapor varied somewhat, increasing (but not proportionally) as the solubility of the solute in water increased. With citric acid added to the aqueous alcohol mixture, the water content (66 percent) of the pervaporated vapor was the same as that of the mixture without added solute. However, in the presence of the solutes

Table 1. Relationship of solute solubilities (*6*) to percentage of water in pervaporated vapor.

Solute	Solubility (g/100 g solvent)		Water in transmitted vapor (%)
	In water	In ethanol	
Control			66
Class I			
Benzoic acid	0.29 (20°C)	52.5 (20°C)	59
Hydroquinone	7.2 (20°C)	49.8 (20°C)	64
Citric acid	163.4 (25°C)	62.1 (25°C)	66
Class II			
Dextrose	97.6 (23°C)	0.22 (23°C)	81
Sodium chloride	36.0 (20°C)	0.07 (18.5°C)	82
Sodium citrate	92.7 (25°C)	0 (25°C)	96

Table 2. Effect of temperature and solute on the pervaporation rates of water and ethanol.

Pervaporating liquid	Temperature (°C)	Pervaporation rate (g/in.² hr)			Water (% in pervaporated vapor)
		Water	Ethanol	Total	
Water	25	0.42		0.42	
	45	1.31		1.31	
Ethanol	25		0.08	0.08	
	45		0.49	0.49	
1/1 Water-ethanol	25	0.32	0.34	0.66	48.5
	45	0.88	0.45	1.33	66.2
1/1 Water-ethanol (with 2 percent sodium citrate)	25	0.25	0.01	0.26	96.1
	45	0.81	0.02	0.83	97.6
1/1 Water-ethanol (with 2 percent benzoic acid)	25	0.15	0.24	0.39	38.5
	45	0.74	0.53	1.27	58.3

that are only moderately or slightly soluble in water, the water content of the pervaporated vapor was less than that of the control, being 64 and 59 percent for hydroquinone and benzoic acid, respectively.

Class-II solutes included dextrose, sodium chloride, and sodium citrate. The percentage of water in the transmitted vapor was much greater in every case with these latter solutes present than that in the control, being 81 to 82 percent for dextrose and sodium chloride and 96 percent for sodium citrate. In the presence of the class-II solutes, the percentage of water in the transmitted vapor apparently increases with progressive insolubility of the solute in ethanol.

A chamber was built to obtain more precise data related to the solute and temperature effects on the pervaporation rates of water and alcohol. It consisted of an aluminum cylinder 1 in. long with an inside diameter of 3.5 in., which was closed with membranes at the ends. Two aluminum rings with gaskets attached were used to clamp the cellophane membranes in liquid-tight seals. Two holes were provided in the wall of the cylinder, one for a reservoir tube and the other for a thermometer.

Experiments were carried out using water, ethanol, 1/1 ethanol-water, a 1/1 ethanol-water solution that contained 2 percent sodium citrate, and a 1/1 ethanol-water solution that contained 2 percent benzoic acid. Two temperatures, 25°C and 45°C, were utilized in a forced-draft oven. The pervaporation chamber was weighed, sampled initially, and sampled again after 3 hours. The percentage of water in the samples was determined by a modification of the Karl Fischer method (*4*). The pervaporating solution was stirred continuously by means of a magnetic stirrer. The data obtained are presented in Table 2.

When ethanol and water are pervaporated separately, the ethanol-pervaporation rate increases more rapidly with a rise in temperature than does the water rate. However, when a 1/1 mixture of the two are pervaporated, different results are obtained. At the lower temperature, the presence of water enhances the passage of the ethanol, increasing its pervaporation rate from 0.08 to 0.34 g/in.² hr (Table 2). At the higher temperature, the rate of ethanol is approximately the same (0.49 to 0.45 g/in.² hr), regardless of whether water is present. The net effect of the temperature increase from 25° to 45°C, when a 1/1 aqueous ethanol solution is pervaporated, is to increase the percentage of water in the pervaporated vapor from 48.5 to 66.2.

The presence of 2 percent sodium citrate (water-soluble, ethanol-insoluble) in a 1/1 solution of ethanol and water decreases the ethanol pervaporation rate to a barely detectable level but decreases the water rate only slightly. The net effect is to increase the water content of the pervaporated vapor from 48.5 percent (control) to 96.1 percent, when one is operating at the lower temperature, and from 66.2 percent (control) to 97.6 percent at the higher temperature (Table 2).

The net effect of benzoic acid (water-insoluble, ethanol-soluble) is opposite to, but less pronounced than, that of sodium citrate. The water rate is reduced considerably, while the ethanol rate is reduced only slightly at the lower temperature and is even increased at the higher temperature. The net effect is a decrease in the percentage of water in the pervaporated vapor from 48.5 to 38.5 percent at 25°C and from 66.2 to 58.3 percent at 45°C (Table 2).

From thermodynamics, one would expect that the presence of a water-soluble, ethanol-insoluble solute would enhance the evaporation of ethanol from a water-ethanol mixture. An experiment involving evaporation from an open dish (no membrane) was conducted at 25°C, in which mixtures of equal parts of ethanol and water, with and without 2 percent sodium citrate, were evaporated for 6 hours in a forced draft (*5*).

As expected, the addition of sodium citrate resulted in a slight reduction of the water-evaporation rate (from 0.07 to 0.05 g/in.² hr) and a slight increase in the ethanol rate (from 1.05 to 1.10). Thus, interposition of a membrane at 25°C with sodium citrate present reversed the composition of the vapor from 96 percent ethanol to 96 percent water (Table 2). The total pervaporative rate of aqueous alcohol without solute present at 25°C (Table 2) was about half the total evaporative rate of the same mixture without a membrane.

The "solute effect" must be due to interfacial phenomena. The following explanation is postulated. As the solution is pervaporated, solute molecules either adhere to, or become trapped in, the membrane. Probably the latter is the case, since the solution is stirred vigorously during the pervaporation. The "good" solvent, by means of interaction with the solute molecules, can readily pass through the membrane. The "poor" solvent is repelled from the solute molecules, and its passage through the membrane is thus restricted.

If this hypothesis is correct, then a membrane impregnated with solute should give a "solute effect" until the solute is gradually dissolved from the membrane if it is used in the pervaporation of a water-ethanol mixture. Conversely, if the solute is in the mixture and a clean membrane is used, the "solute effect" should increase with time. This was shown to be the case in two experiments. First, a membrane soaked overnight with a saturated aqueous solution of sodium citrate was used to pervaporate a 1/1 solution of aqueous ethanol. After 2 hours the water content of the pervaporated vapor was 82 percent, and at 4 hours it had dropped to 64 percent. Then a 1/1 aqueous ethanol solution containing 2 percent sodium citrate was pervaporated at 45°C for 8 hours, with hourly sampling. The ethanol rate, which was very low at the beginning, was reduced to zero by the third hour.

E. G. Heisler, Ann S. Hunter, James Siciliano, R. H. Treadway
Eastern Regional Research Laboratory, Eastern Utilization Research Branch, Agricultural Research Service, U.S. Department of Agriculture, Philadelphia, Pennsylvania

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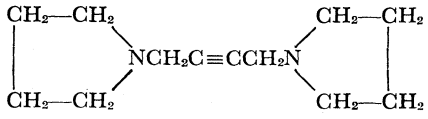
Tremor Induced by Tremorine and Its Antagonism by Anti-Parkinson Drugs

During the course of screening drugs in mice, it was observed that 1,4-dipyrrolidino-2-butyne (Tremorine)



produces a profound tremor of the head and limbs lasting for more than 1 hour. In addition, the animals move about slowly, show rigidity, and are less active. Parasympathetic stimulation is marked and characterized by profuse salivation, miosis, diarrhea, and bradycardia. A profound fall of body temperature also occurs. Sustained tremor is a rare phenomenon of drug action (in our experience, less than 10 of 10,000 drugs tested).

Because of the possible usefulness of such a drug in testing for anti-Parkinson agents, further studies have been carried out. In mice, doses of 20 mg/kg given orally, intraperitoneally, intravenously, or subcutaneously are effective, the tremors appearing within 10 to 30 minutes, depending on the route, and lasting 3 to 4 hours. Such drugs as atropine, scopolamine and other anti-Parkinson agents control the tremor and cholinergic effects completely in one dose of 2 to 10 mg/kg. They are effective either given prior to Tremorine or after full tremor effects are evident. Methantheline will control the parasympathomimetic effects but does not affect the tremor; thus the central and peripheral actions of the drug can be separated. Central depressants such as the barbiturates, mephenesin, alcohols, anticonvulsants, and analgesics are ineffective against the tremor in doses less than those causing marked ataxia, sedation, or hypnosis. Hexamethonium and TEA were also ineffective. These results emphasize the specificity of the antagonism of Tremorine tremor by the anti-Parkinson drugs in contrast to nicotine tremor which is ephemeral, has both central and peripheral components, and may be controlled by a wide variety of agents (*1*).

Tremorine in dogs has effects similar to those observed in mice. In the dog, a dose of 5 mg given intraperitoneally gives a full picture of Tremorine action. In the monkey, tremor is not as marked as it is in dogs, but it is easily seen and the Parkinsonlike facial changes are very striking. Tremorine action in the dog and monkey develops within the first hour. The tremors last for 24 hours or more and parasympathetic stimulation lasts for 2 or 3 days. Repeated doses of atropine or other anticholinergic agents will control these effects. Tremorine is often fatal in dogs and monkeys because of respiratory complications if adequate treatment is not given.

Twenty analogs of Tremorine were tested. None produced tremor. This suggests a high degree of chemical specificity for the production of these effects.

The site of action of Tremorine is under investigation. The tremor occurs in decerebrate rats, mice, and rabbits. Tremor also appeared in a chronic completely decerebellate dog, superimposed on the much coarser and slower tremor normally present in this animal (*2, 3*).

G. M. Everett
L. E. Blockus
I. M. Shepperd
Department of Pharmacology, Abbott Laboratories, North Chicago, Illinois

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7 March 1956

Another Case of Anomalous Pregnancy in a White Rat

Evans (*1*) reported a few specific instances of anomalous pregnancies in rodents and suggested that the phenomenon might be more common than a review of relevant literature would indicate. In four unequivocal cases (one in a rat, two in mice, and one in a guinea pig), the animals, although they were segregated from the time that pregnancy was observed until the time that the young were of weaning age, delivered a second litter.

In our laboratory, female rats are invariably isolated in heavily meshed individual cages as soon as pregnancy is observed. A case of anomalous pregnancy was noted in 1955, but detailed records are not available. However, another unequivocal case of anomalous pregnancy was observed and recorded in 1956. A healthy seven-pup litter was delivered by albino rat XX (Wistar strain) on 21 Jan. 1956, each member of which has survived to date. On 11 Feb. 1956, this rat produced a healthy eight-pup litter, each member of which was still alive at 16 days of age. The first litter consisted of three males and four females, and the second consisted of three males and five females.

Thus, 23 days elapsed between the production of the first and second litters. For at least 6 days before the birth of the first litter, rat XX was securely isolated, and from parturition until the birth of the second litter, it was segregated from all except its own immature offspring. With these, it shared a cage until 2 days before the birth of the second litter.

Evans discusses critically two theories explanatory of such anomalous pregnancies, namely, delayed implantation at the blastocyst stage, which is said to occur sporadically in rodents, and superfetation. Evans thought that the superfetation hypothesis was unlikely in the case of her rat, since the interval between its litters (25 days) was greater than the average gestation period. If the essential criterion for superfetation is a shorter-than-normal gestation period, the superfetation hypothesis does not seem relevant to the case of our rat, where the 23-day interval between the delivery of litters represents, according to Farris (*2*), a gestation period that is well within the normal range.

If the delayed implantation hypothesis were invoked in the case of rat XX, it would have to be assumed that the blastocysts had survived during the first pregnancy, been retained in the uterus during labor, and implanted after delivery of the first litter. Boyd and Hamilton (*3*) discuss lengthened gestation in lactating rodents in relation to physiological delay of implantation, but, in the case of our rat, the period seems improbably prolonged.

Another possibility is tentatively suggested. Rat XX was segregated 6 to 7 days before the birth of the first litter, and the total time between segregation and the birth of the second litter was thus 29 to 30 days. Farris (*2*) says that gestation may be extended a week or more if a female is carrying a large number of young and is suckling a litter. Thus, if it were possible for a pregnant female to be inseminated, this may have occurred just before segregation, and the 29 to 30-day gestation period does not constitute an improbable gestation period for a multiparous rat that is suckling a seven-pup litter. Although the sizes of the litters renders this explanation unlikely, the remote possibility that this rat may have been fertilized by way of the other uterine horn is nevertheless suggested for consideration.