In the present work, the influence of chilling temperatures on streaming was observed in cells of young petiole trichomes of several species. Observation of individual cells was possible by moving the microscope, mount, and light, as a unit from one constant-temperature room to another. A fan blew air across the stage to insure rapid temperature equilibrium and to reduce heating of the subject from the illumination. In all the chilling-sensitive plants observed—tomato (*Lycopersicon esculentum*), watermelon (*Citrullus vulgaris*), honeydew melon (*Cucumis melo*), a tobacco (*Nicotiana glutinosa*), and sweetpotato (*Ipomoea batatas*)—streaming ceased or was just perceptible after 1 or 2 minutes at 10°C. Invariably it ceased promptly at 5° or 0°C. In contrast, streaming in the chilling-insensitive plants observed—radish (*Raphanus sativus*), carrot (*Daucus carota*), and filaree (*Erodium cicutarium*)—proceeded at 0° or 2.5°C.

For tomato trichomes, increasing the exposure at 0°C delayed increasingly the resumption of streaming on transfer to 20°C. If exposure to 0°C exceeded about 24 hours, streaming failed to resume in most trichomes. In contrast, streaming in filaree proceeded slowly but steadily at 0°C for up to 3 days. On transfer back to 20°C, rapid streaming was resumed in 1 or 2 minutes.

In addition to these effects on streaming, chilling caused other responses. After 2 days of chilling at 0°C, some trichome cells of tomato were plasmolyzed. In others, the protoplasts formed into aggregated clumps, and small particles executed Brownian movement in the cell sap. Such movement was never observed in unchilled trichomes. This structural disorganization of the protoplast by chilling may result from syneresis of the protoplasmic gel, as has been suggested for a slime mold (*3*).

The striking difference described here between chilling-sensitive and chilling-insensitive plants suggests an association between susceptibility to chilling injury and cessation of streaming. Chilling injury and cessation of streaming may be separate symptoms of some more fundamental disorder that is induced at 10°C or below in chilling-sensitive plants. Alternatively, the visible symptoms of chilling injury could be effects secondary to cessation of streaming.

The contrasting effect of chilling temperatures on streaming in cells of sensitive and insensitive plants may reside in a differential effect of temperature on one or more of the following: cell lipids (*4*) and their role in structure and action of the protoplasm; energy supply from respiration to maintain protoplasmic streaming; energy utilization for streaming; or protoplasmic viscosity. If the mechanism of streaming is related to an adequate supply of adenosine triphosphate (*5*), some interesting comparative biochemistry for chilling-insensitive and chilling-sensitive plants is suggested.

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Direct-Reacting Bilirubin, Bilirubin Glucuronide, in Serum, Bile, and Urine

Since the introduction into clinical chemistry of diazotized sulfanilic acid for the determination of serum bilirubin, it has been recognized that bilirubin occurs in two different forms, one giving a so-called "direct" reaction, the other exhibiting a so-called "indirect" reaction in which coupling takes place only after the addition of alcohol (*1*). In general, when the jaundice is due to excessive destruction of hemoglobin, as it is in hemolytic anemia, most of the bilirubin in serum is of the indirect-reacting form. If the jaundice results from intrahepatic or extrahepatic obstruction to the passage of bile into the intestine, most of the bilirubin in serum is of the direct-reacting form.

Various explanations have been offered to account for this clinically important difference, but the experimental evidence in support of these theories has been controversial (*2*). Recent studies have shown that direct-reacting bilirubin obtained from serum or bile is water-soluble over a wide *p*H range, whereas the indirect-reacting pigment is practically insoluble in water below a pH of 8 (*3*). It has been suggested that the solubility in water of the direct-reacting fraction may be the result of a conjugation of bilirubin with a polar substance (*1, 4*). Because of their instability, satisfactory chromatographic purification of these pigments, particularly the direct-reacting fraction, has not been achieved (*4*).

This difficulty has now been overcome by studying (*5*) the more stable dipyrromethene diazonium pigments, which were obtained by treating serum and urine from jaundiced patients or normal bile with an excess of diazotized sulfanilic acid that was dissolved in dilute hydrochloric acid. In this reaction 1 mole of bilirubin reacts with 2 moles of the reagent, yielding 2 moles of hydroxypyrromethene diazonium salt (*6*).

After preliminary purification and butanol extraction, the azo-pigments obtained from serum, bile, and urine were separated and purified by ascending paper chromatography, employing a solvent system that consisted of 75 parts ethyl methyl ketone, 25 parts *n*-propionic acid, and 30 parts water. By using this system, it was found that direct-reacting bilirubin in the serum of jaundiced patients and almost all of the bilirubin in fresh bile and in the urine of jaundiced patients gave rise to an azo-pigment (B) exhibiting an R_f value of 0.25 to 0.30. On the other hand, the azo-pigment (A) obtained from indirect-reacting serum bilirubin, crystalline bilirubin, and heated bile exhibited an R_f value of 0.45 to 0.50. Diazonium salts of synthetic neoxanthobilirubinic acid and isoneoxanthobilirubinic acid (*7*) were found to have an R_f value that is identical with that of indirect-reacting serum bilirubin and that of crystalline bilirubin. Absorption spectra in the visible range appeared to be identical for azo-pigments A and B.

Hydrolysis of repeatedly chromatographed azo-pigment B in 1*N* hydro-

Table 1. Acid and enzymatic hydrolysis of conjugated hydroxypyrromethene diazonium salt (azo-pigment B). (Values are given in micromoles.)

Substrate azo-pigment	Type of hydrolysis	Product	
		Azo-pigment	Glucuronic acid
B, 0.41	1*N* HCl, 1 hr at 100°C	A, 0.41	0.39
B, 0.10	β-glucuronidase*	A, 0.083	0.09
B, 0.70	β-glucuronidase†	A, 0.61	0.58
B, 0.23	β-glucuronidase† (heat-inactivated)	B	< 0.01
B, 0.36	Concentrated sulfuric acid	Destroyed	0.38
A, 0.55	1*N* HCl, 1 hr at 100°C	A	< 0.01

* Beef liver β-glucuronidase (Warner-Chilcott), 24 hr at 37°C and *p*H 4.6.
† Bacterial β-glucuronidase (Sigma, Lot No. 125–63), 24 hr at 37°C and *p*H 6.2.

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.

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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