conscious selection may be carried on.

The findings present an interesting picture of reproduction of long-day, temperature zone plants introduced to the tropics. It is shown that low night temperatures, or changes of temperature associated with high elevations, constitute a mechanism for inducing flowering. The ability of such plants to persist is, therefore, apparent (*5*).

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References and Notes

1. F. W. Went, *The Experimental Control of Plant Growth* (Chronica Botanica, Waltham, Mass., 1957), p. 343.
2. A. C. Leopold, *Quart. Rev. Biol.* **26**, 247 (1951).
3. R. H. Roberts and B. E. Struckmeyer, *J. Agr. Research* **56**, 633 (1938); **59**, 699 (1939).
4. W. M. Hiesey. *Am. J. Botany* **40**, 205 (1953).
5. This study was supported, in part, by Western Regional Technical Committee W-58. Published with the approval of the director, Hawaii Agricultural Experiment Station, as miscellaneous paper No. 113.

21 August 1959

Mammalian Liver β-Glucuronidase for Hydrolysis of Steroidal Conjugates

Abstract. Although the rate of hydrolysis by mammalian β-glucuronidase appears to be inhibited by methylene chloride or carbon tetrachloride with the standard technique (phenolphthalein glucuronide as a substrate), the release of steroidal conjugates under conditions generally employed does not appear to be affected.

Gautney *et al.* (*1*) have recently described the potentiating effect of several organic solvents (aromatic hydrocarbons, chlorinated aliphatic hydrocarbons, aliphatic alcohols) on bacterial β-glucuronidase, thus extending an earlier report concerning chloroform (*2*). The authors, however, found that dichloromethane and chloroform inhibit the hydrolysis of phenolphthalein glucuronide by mammalian liver enzyme. This raises the question whether mammalian liver β-glucuronidase is suitable for hydrolysis of steroidal glucuronides in biological fluids which have previously been extracted with such solvents.

Table 1. Effect of solvents on hydrolysis by liver β-glucuronidase of various glucuronides.

Enzyme units/ml	Control (No solvent)	Dichloromethane (% Aglycon)	Carbon Tetrachloride (% Aglycon)
	Phenolphthalein		
6.25	2.2	1.0	2.8
12.5	5.4	2.2	4.9
25.0	15.0	4.7	12.7
50.0	32.0	9.7	30.0
250.0	69.0	54.0	71.5
1000.0	100.0	98.0	100.0
	17-Ketosteroids		
10.0	16.0	9.4	10.7
100.0	45.2	43.3	41.0
500.0	100.0	96.0	94.0
	Corticoids		
10.0	16.0	14.0	13.3
100.0	58.5	67.5	62.3
250.0	85.5	81.3	81.0
500.0	100.0	99.5	100.0

Previous studies in our laboratory had demonstrated quantitative release of the free steroid when pregnane-3α, 17α, 21-triol-11, 20-dione monoglucuronide, added to human plasma previously extracted with dichloromethane, was incubated with 300 to 500 Fishman units of mammalian β-glucuronidase per milliliter (*3*). In view of the implications of Gautney's (*1*) results, this matter was reinvestigated.

A pool of human urine was divided into aliquots of 10 ml, and duplicates were equilibrated with either dichloromethane or carbon tetrachloride. Thereafter the urine was incubated with beef liver β-glucuronidase (*4*) in doses of 10 to 500 Fishman units per milliliter at *p*H 4.5, 37°C for 48 hours. The 17-ketosteroids were extracted and measured as reported elsewhere (*5*), and the urinary corticoids were determined by the method of Silber and Porter (*6*). In addition, the mammalian enzyme was studied by use of phenolphthalein glucuronide as substrate after the manner of Talalay *et al.* (*7*), with the addition of 0.1 ml of the organic solvent to replicate digestion mixtures employed by Gautney (*1*) in his investigation.

The results (Table 1) are expressed as percentage of maximal hydrolysis achieved with each substrate. In agreement with Gautney *et al.* (*1*), the solvents inhibited hydrolysis of phenolphthalein glucuronide by the low concentrations of enzyme often employed. On the other hand, under the conditions of incubation with high enzyme concentrations for 48 hours, as generally described for hydrolysis of steroid conjugates (*3, 8*), no significant inhibition was apparent. At lower concentration of enzyme the solvents only slightly reduced the partial hydrolysis of steroid glucuronides. These results do not support the assertion by Gautney *et al.* (*1*) that such solvents must not be used for prior extraction if liver β-glucuronidase is to be employed for subsequent hydrolysis of steroid glucuronides.

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References

1. M. C. Gautney, S. B. Barker, S. R. Hill, Jr., *Science* **129**, 1281 (1958).
2. Sigma Chemical Co., "Urgent bulletin re bacterial glucuronidase" (St. Louis, Mo., Nov. 1958).
3. A. M. Bongiovanni and W. R. Eberlein, *Proc. Soc. Exptl. Biol. Med.* **89**, 281 (1955).
4. Ketodase (Warner-Chilcott).
5. A. M. Bongiovanni, W. R. Eberlein, P. Z. Thomas, *J. Clin. Endocrinol. and Metabolism* **17**, 331 (1957).
6. R. H. Silber and C. C. Porter, in *Methods of Biochemical Analysis*, D. Glick, Ed. (Interscience, New York, 1957), vol. 4, p. 139.
7. P. Talalay, W. H. Fishman, C. Huggins, *J. Biol. Chem.* **166**, 757 (1946).
8. D. K. Fukushima and T. F. Gallagher, *J. Clin. Endocrinol. and Metabolism* **18**, 694 (1958); A. M. Bongiovanni and W. R. Eberlein, *Anal. Chem.* **30**, 338 (1958); E. M. Glenn and D. H. Nelson, *J. Clin. Endocrinol. and Metabolism* **13**, 911 (1953).

1 July 1959

Glucuronidase Activation: Enzyme Action at an Interface

Abstract. The potentiating action of chloroform on bacterial β-glucuronidase has been shown to increase as the interface area between the two liquid phases increases. Prior extraction of the enzyme with chloroform causes a loss rather than an increase in activity. It is tentatively suggested that the correlation between activity and interface area may reflect a phenomenon of enzyme action at a liquid/liquid interface.

In an investigation of the "paradoxical" effect of organic solvents on the activity of animal and bacterial β-glucuronidase, which was first observed with chloroform (*1*), and more recently with other solvents by Gautney, Barker, and Hill (*2*), we have also observed an activation of the bacterial enzyme (*3*) varying in degree with the solvent used and a similar inactivation of the animal enzyme (*4*). We have further noted that the activation of the bacterial enzyme seems not to be due to the removal of an inhibitor, since prior extraction of the enzyme solution by shaking with one volume of chloroform for 5 minutes causes a 40 percent loss rather than an increase in activity. An activation such as that noted by Bernfeld, Jacobsen, and Bernfeld (*5*) has been ruled out by the work of Gautney, Barker, and Hill. Moreover, the continuous removal of phenolphthalein from the reaction site cannot account for the inverse effect on the animal and bacterial enzymes. These facts suggest that some physicochemical property of these enzymes is involved in the activation by solvents. The preliminary results reported here relate to this problem.

The assay method used in this study is that suggested by the Sigma Chemical

Table 1. Effect of varying the interface area on the activation of bacterial β-glucuronidase by a constant volume of chloroform. Assay mixture, 0.5 ml of 0.2-percent glucuronidase; 0.5 ml of 0.075*M* phosphate buffer, *p*H 6.8; and 0.5 ml of 0.015*M* substrate. Volume of chloroform, 1 ml.

Interface area (approx.) (cm^2)	Activity (per gm)
0	25,500
0.38	61,500
0.95	78,000
2.0	88,000

Table 2. Activation of bacterial β-glucuronidase by 0.1 ml of chloroform present as a layer and as an emulsion. Assay mixture as in Table 1; incubation in 15 × 125-mm test tubes.

Condition of chloroform	Activity (per gm)
None	25,500
Layer	71,000
Emulsion (a)*	81,000
Emulsion (b)*	95,000

* The chloroform was more finely divided in (b) than in (a).

Co. (*6*) and is based on that of Talalay, Fishman, and Huggins (*7*). Phenolphthalein glucuronide is used as substrate, but incubation is in 0.075*M* phosphate buffer at *p*H 6.8. Action of the enzyme is stopped by the addition of 0.2*M* glycine buffer at *p*H 10.4. In our experiments the tubes containing the assay mixture and the solvent were restoppered after the addition of the glycine buffer and then shaken vigorously and centrifuged in order to ensure equilibration of phenolphthalein between the solvent and the alkaline aqueous phase.

When a constant volume of chloroform is incubated with the assay mixture in vessels of different dimensions, so as to vary the interface area between the two liquids, there is an increasing activation of the enzyme as the interface area increases (Table 1). This same increase in activation with a constant volume of chloroform can be achieved by merely shaking the vessel prior to incubation so as to emulsify the chloroform and thus increase the interface area (Table 2). When the vessel is thus shaken, the extent of activation is dependent on the degree of visible emulsification. Conversely, if the interface area is maintained constant while the volume of chloroform is varied, the degree of activation of the enzyme remains constant and independent of the volume of chloroform. Thus, when the assay mixture is layered over volumes of chloroform varying from 5 to 13 ml, with an interface area of 0.38 cm^2, the activity averaged 45,000 (range 42,000 to 47,000)—that is, less than 50 percent of that produced by 0.1 ml of chloroform present as an emulsion. The fact that this value is somewhat less than that for the same interface area in Table 1 is due to the day-to-day variation often observed in the assay of glucuronidase activity (*1*). However, its constancy with regard to the variations seen in Table 1 emphasizes that the interface area is the determinant.

These in vitro results suggest an action of the enzyme at the solvent/water interface and could be a reflexion of a fundamental phenomenon of enzyme action at lipid/water interfaces in the cell, as suggested by Robertson (*8*) and more recently by Danielli and Davies (*9*). This effect probably depends on some special physicochemical property of the bacterial enzyme not shared by the animal enzyme and may involve the concentration and possibly the orientation of the enzyme at the interface. The effect differs from that noted by Schulman (*10*), who attributed the difference in reaction rate in the hydrolysis of various esters by pancreatin to orientation of the substrate molecules at the ester/water interface. Wasteneys and Borsook (*11*) showed that the activity of pepsin in the synthesis of plasteins was increased by the addition of benzene or benzaldehyde. The observation by Doyle (*12*) that *Escherichia coli* glucuronidase can be split into two fragments, (i) an inactive butyl-alcohol-soluble fragment of undefined nature and (ii) a much less active protein fragment, is suggestive of a lipoprotein character for the enzyme and would not be incompatible with the notion of concentration at the solvent-water interface. Finally, in addition to this interface effect observed by us, the nature of the solvent plays an important role, as is seen in the varying activating power of different solvents observed by Gautney, Barker, and Hill and confirmed by us.

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References and Notes

1. "Urgent Bulletin re Bacterial β Glucuronidase" (Sigma Chemical Co., St. Louis, 1958).
2. M. C. Gautney, S. B. Barker, S. R. Hill, *Science* **129**, 1281 (1959).
3. The bacterial enzyme used in this study was obtained from the Sigma Chemical Co., St. Louis, Mo.
4. The animal enzyme was obtained from Viobin Laboratories, Monticello, Ill.
5. P. Bernfeld, S. Jacobson, H. C. Bernfeld, *Arch. Biochem. Biophys.* **69**, 198 (1957).
6. *Sigma Chemical Co. Bull. No. 105* (1951).
7. P. Talalay, W. H. Fishman, C. Huggins, *J. Biol. Chem.* **166**, 757 (1946).
8. T. B. Robertson, *Australian J. Exptl. Biol. Med. Sci.* **3**, 97 (1926).
9. J. F. Danielli and J. T. Davies, *Advances in Enzymol.* **11**, 35 (1951).
10. J. H. Schulman, *Trans. Faraday Soc.* **48**, 134 (1941).
11. H. Wasteneys and H. Borsook, *Colloid Symposium Monograph* **6**, 155 (1928).
12. M. L. Doyle, *Federation Proc.* **13**, 201 (1954); ——— and P. Katzman, *ibid.* **11**, 203 (1952).

12 October 1959

Characterization of Endogenous Ethanol in the Mammal

Abstract. Ethanol has been isolated from the tissues of several animal species in amounts ranging from 23 to 145 μmole/100 gm of tissue. Intestinal bacterial flora appear to be excluded as a source of this ethanol. Radioactivity from pyruvate-2-C^{14} appeared in ethanol after incubation with liver slices; this finding indicates an endogenous synthesis.

Whether ethanol occurs endogenously in mammalian tissues has been a moot question for years. Although some investigators (*1*) have reported the presence of ethanol in amounts of from 2 to 5 mg per 100 gm of tissue or per 100 ml of plasma, as determined by chemical methods, other investigators (*2*) have questioned this finding on the basis of possible bacterial contamination of the tissues studied or inadequate isolation and assay procedures. This report describes some experiments designed to clarify this question.

Liver, kidney, heart, and skeletal muscles were removed rapidly from animals and homogenized immediately in a one-to-one mixture of ice-cold distilled water or 0.1*M* phosphate buffer at *p*H 7.4 in a Potter-Elvehjem homogenizer. The skeletal and heart-muscle samples were minced prior to homogenization. In some cases the homogenate was heated for 5 minutes in a water bath at 80° to 90°C in a sealed tube and then freeze-dried in a glass lyophilizer. In other cases there was no heating. The lyophilates were kept frozen until it was time to assay them for ethanol. Aliquots of the lyophilate were assayed by spectrophotometric measurement of diphosphopyridine nucleotide (DPN) reduction catalyzed by twice-recrystallized yeast alcohol dehydrogenase (*3*) according to the procedure described by Bonnichsen and Theorell (*4*).

In vitro formation of ethanol was studied in liver slices which were incubated in Warburg flasks in an atmosphere of nitrogen at 38°C; about 300 mg of slices per milliliter of Krebs-Ringer phosphate medium were used. At the end of these experiments, the tissues and medium were prepared for analysis in the manner specified above, except that heating was omitted.