methylation. It appears in the urine in a combined form which could be a di- or a mono-etheral sulphate (cf. adrenaline; Richter, 1940; Deichmann, 1943).

The preparation of anisoylglucuronide has not been described before, but Quick (1932) has reported that the corresponding acid, anisic acid, gives rise in the dog and in man to a glucuronide which was not isolated and to a glycine conjugate, *p*-methoxyhippuric acid, which was isolated.

SUMMARY

The fate of veratraldehyde of veratric acid and of anisaldehyde in the rabbit has been studied:

(1) In the case of veratraldehyde, about 70 % of the dose can be accounted for as veratric acid (c. 28 %) and veratroylglucuronide (c. 38 %). There is some uncertainty regarding the relative amounts of these two metabolites owing to the instability of the glucuronide in rabbit urine.

(2) About 70 % of veratric acid can also be accounted for, about 41 % as free veratric acid and about 28 % as its glucuronide.

(3) The results suggest that the amount of glucuronide formed from the aldehyde is greater than from the acid. The implications of this suggestion are discussed.

(4) A minor metabolite of both veratric acid and aldehyde was identified as catechol, which was responsible for the small increase in etheral sulphate output. Vanillic acid derivatives, veratroyl-glycine and protocatechuic acid were not found in the urine.

(5) Veratroylglucuronide and some of its derivatives have been prepared and some of their properties described.

(6) Anisoylglucuronide has been prepared from the urine of anisaldehyde-fed rabbits and some of its derivatives have been described.

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The Esterification of Glycerol with Hippuric Acid and the Behaviour of the Resulting Product towards Enzymes

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The present paper is a report on the first of a series of ester compounds of glycerol with amino-acid derivatives which are being synthesized in order to investigate their chemical properties and behaviour towards enzymes. The synthesis of esters of the amino-acids with glycerol has been twice reported. Abderhalden & Guggenheim (1910) obtained the ester of tyrosine with glycerol and later Fodor & Weizmann (1926) and Weizmann, Haskellberg & Malkova (1929) (cf. Haskellberg, 1930) prepared the mono-ester of glycerol with leucine. Though other esters of glycerol with amino-acids (alanine and glycine esters) were prepared by

Fodor & Weizmann (1910) crystallization of these compounds presented great difficulty and in the case of the alanine and glycine esters was unsuccessful.

This paper describes an attempt to obtain a crystalline product from glycerol and glycine by first substituting the free amino group of glycine with the benzoyl radical as in hippuric acid. The behaviour of the resulting ester towards lipases and esterases was investigated, but this could not be done in the case of the leucine ester owing to its extreme instability in aqueous solution. Preliminary work on the esters of glutamic acid and

pyrrolidone carboxylic acid with glycerol has shown that the former did not crystallize, although a saponifiable fraction was obtained, whereas the latter gave a crystalline product.

EXPERIMENTAL

(With the collaboration of Miss S. Bermann)

Preparation of hippurylglyceride

Whereas Fodor & Weizmann (1926) reported a strongly exothermic reaction when the sodium salts of the amino-acids employed were mixed with glycerol chlorohydrin by grinding in a mortar, this was not observed with the sodium salt of hippuric acid and glycerol chlorohydrin. Higher temperatures were therefore employed.

After thorough mixing of 30 g. of the sodium salt of hippuric acid with an equivalent amount of glycerol chlorohydrin in a porcelain mortar, the mixture was transferred into a small Erlenmeyer flask fitted with a condenser. The mixture, kept in an oil bath at 155° for an hour, appeared as a dark syrup, which was dissolved in a small quantity of absolute ethanol and filtered from the sodium chloride formed during the esterification. The alcoholic solution was poured into a large excess of ether, whereupon a yellow syrup separated. After standing overnight, the mother liquor was discarded and the syrup was dissolved in about 6 ml. acetone and poured into 100 ml. of light petroleum. A whitish emulsion was obtained, from which, after several hours, drops of a yellowish syrup separated. After standing overnight, the mother liquor was discarded and the semi-crystalline layer was redissolved in acetone and again precipitated by the addition of light petroleum. After this procedure had been repeated several times all the material was obtained crystalline. It was then washed with light petroleum and dried in a vacuum desiccator over sulphuric acid and paraffin. Yield: 6 g. For all analyses and enzyme tests this product was recrystallized twice from absolute ethanol by addition of ether. (Recrystallization from acetone by precipitation with light petroleum gave material yielding unsatisfactory elementary analyses.) It should be noted that once the product has been obtained crystalline, it may be recrystallized very easily. It is insoluble in ethyl acetate, ether, light petroleum or cold water and dissolves easily in hot acetone or ethanol. For analysis it was dried to constant weight over P_2O_5 at the b.p. of chloroform. The dry crystalline substance, m.p. 130° (uncorr.), was free of chloride and other ash. (Found: C, 57.1; H, 5.9; N, 5.4%. $C_{12}H_{15}O_5N$ requires C, 57.1; H, 5.9; N, 5.5%.)

Saponification value. The product (31.5 mg.) was dissolved in a small amount of hot water, a few drops of phenolphthalein were added and sufficient 0.02N-NaOH to produce a weak pink colour (0.1–0.15 ml.). The solution was then heated to boiling and 0.02N-NaOH was added drop by drop from a microburette until the pink colour remained permanently. Excess of NaOH was avoided in order to prevent the hydrolysis of the benzoyl-glycine linkage. (It was, however, established that this did not result even from addition of several ml. 0.02N-NaOH and prolonged boiling.) Saponification value: 31.5 mg. required 6.2 ml. 0.02N-NaOH. Calc. 6.3 ml.

Enzyme tests

Owing to the considerable lability of the ester in alkaline or acid medium, the tests could be conducted only with pancreatic lipase,* which is fairly active at pH 7. Phosphate buffer of pH 7 was employed.

Another difficulty was encountered in that the ester of glycerol with hippuric acid is insoluble in cold water or phosphate buffer. Its solution in a small quantity of ethanol, poured into phosphate buffer—enzyme mixture, formed a colloidal suspension, stabilized probably by the protein of the pancreatic extract. The same amount of ethanol was added to the controls.

Table 1. *Cleavage of hippurylglyceride by a glycerol extract of pancreatin activated by sodium taurocholate*

Solutions.

(1) 0.1023 g. hippurylglyceride dissolved in 1.1 ml. ethanol, 12 ml. phosphate buffer (Sorensen, pH 7), 0.9 ml. 1.5% sodium taurocholate, 3 ml. 33% glycerol extract of pancreatin, water to 22 ml.

(2) The same as (1) but 0.9 ml. water instead of 0.9 ml. sodium taurocholate.

(3) The same as (1) but with 3 ml. water instead of 3 ml. pancreatin extract.

(4) 1.1 ml. ethanol, 12 ml. phosphate buffer, 0.9 ml. sodium taurocholate solution, 3 ml. pancreatin extract, water to 22 ml.

(5) As (4), with 0.9 ml. water instead of 0.9 ml. sodium taurocholate.

Owing to the presence of phosphate ions, Ca-salts could not be used as activators. The use of veronal buffer, on the other hand, was excluded by its strongly inhibitory action on pancreatic lipase (Oppenheimer, 1936).

Result.

5 ml. of each mixture were titrated against 0.02N-NaOH by Sorensen's formol-titration method.

Incubation time (min.)	Titration increase (ml. 0.02N-NaOH)				
	(1)	(2)	(3)	(4)	(5)
90	1.40	1.20	—	0.75	0.55
180	1.90	1.50	—	—	0.25
270	2.30	2.05	—	—	—
Cleavage, corrected for controls	1.50	1.25			

Cleavage in (1) corresponds to 32.6%; in (2) to 27.2% of the weight amount.

No cleavage with liver esterase could be effected. For these experiments pig liver was ground in a mincing machine and mixed well with fuller's earth. Treatment in a Buchner press gave an extract which was diluted with an equal volume of water. The

* Prepared by extraction of pancreatin (Parke Davis and Co.) with 95% glycerol. It was previously established that sodium hippurate is not debenzoylated by this extract.

activity of the extract was such that 75 % of added ethyl butyrate was hydrolyzed in 3.5 hr. incubation time.

SUMMARY

1. The synthesis of an ester of glycerol with hippuric acid from glycerol chlorohydrin and sodium hippurate is described.

2. Cleavage effected by the action of a glycerol extract of pancreatin activated by sodium taurocholate amounted to 32.6 % after 270 min. The cleavage by the same extract in the absence of sodium taurocholate was 27.2 %.

3. Cleavage of hippurylglyceride was not effected by liver esterase.

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Yeast Lactic Dehydrogenase and Cytochrome b_2

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The dehydrogenases fall into three distinct groups, according to the nature of the intermediate hydrogen acceptors through which they react with O_2 (cf. Green & Brosteaux, 1936):

Group I, dehydrogenases which depend on the presence of one or other of the nicotinic amide nucleotides (coenzymes I and II). Through the latter substances, which act as intermediate acceptors or hydrogen carriers, they react with certain flavoproteins and so with cytochrome *c* and O_2 . They cannot, however, react directly with flavoproteins, cytochrome or O_2 , or with artificial acceptors like methylene blue.

Group II, flavoproteins, which do not require the addition of coenzymes, the prosthetic flavin group itself acting as intermediate acceptor. In some cases (aerobic dehydrogenases) this group can react directly with O_2 ; in other cases (diaphorases) the mediation of the cytochrome system is necessary.

Group III, the 'cytochrome-reducing dehydrogenases', so-called because (at any rate in the unpurified state) they readily reduce cytochrome *c*, though this may not be a direct reaction. These enzymes have a number of properties in common which distinguish them from those of the other groups. They do not depend on soluble coenzymes and readily reduce methylene blue in their absence.

Several dehydrogenases of group I have been isolated in the pure state as crystalline proteins. They do not contain any coloured prosthetic group. Several enzymes of group II have been obtained in

a state approximating to purity. None of the enzymes of group III, on the other hand, has previously been isolated or purified to any great extent, and consequently the nature of the active groups of these enzymes is unknown. The reason for this is that almost all the enzymes of this class are attached to the insoluble part of the cell-structure and until they can be detached a high degree of purification is hardly possible.

Among the chief enzymes of group III are the succinic dehydrogenase, the lactic dehydrogenase of yeast (that of animal tissues is a quite distinct enzyme belonging to group I), the insoluble glycerophosphate dehydrogenase of animal tissues (the soluble glycerophosphate enzyme is again a different enzyme of group I) and probably choline dehydrogenase. The yeast lactic enzyme is of special interest because it is freely soluble and therefore its purification and isolation may be attempted with some hope of success. We undertook this with the object of obtaining information on the chemical nature of the enzyme and especially of its prosthetic group, if any, and also of studying the manner in which the purified enzyme reacts with cytochrome and other hydrogen acceptors.

Lactic dehydrogenase was first extracted from yeast by Bernheim (1928), who treated Delft baker's yeast with acetone and extracted the dry residue with alkaline phosphate solutions. Several later workers have failed to obtain active solutions in this way; the reason for this we have found to be that the acetone yeast is usually acid enough to neutralize the alkaline phosphate. If the solution is kept alkaline, Bernheim's results can be repeated.

* Preliminary notes on certain parts of this work have been published in *Nature* by Dixon & Zervas (1939), Bach, Dixon & Keilin (1942) and Bach, Dixon & Zervas (1942).