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The Synthesis of Glucuronides by Liver Slices

By I. D. E. STOREY*
Department of Biochemistry, University of Edinburgh

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Although the formation of conjugated glucuronides in the animal body has been known for many years, surprisingly little has been learned as to the chemical mechanisms involved. Early attempts to determine the origin of the glucuronic acid were carried out on the whole animal, and the results were frequently difficult to interpret (see Williams, 1947), but the researches of Quick (1926) pointed to glycogen, rather than glucose, as the source of the glucuronic acid.

The only extensive investigation of the conjugation process in isolated tissue preparations is that of Lipschitz & Bueding (1939). These authors found that the synthesis of glucuronides, by guinea pig-liver slices in presence of glucuronidogenic substances such as borneol and menthol, was considerably increased by lactate, pyruvate and dihydroxyacetone, but that glucose and glucuronic acid were without any appreciable effect. They further showed that no synthesis took place under anaerobic conditions, or in presence of cyanide, fluoride or iodoacetate, and concluded that oxidative processes and esterification of phosphoric acid were necessary. In the present paper, the synthesis of glucuronides as it occurs in mouse-liver slices has been examined, using the rapid and sensitive method developed by Levvy & Storey (1949).

* Present address: Department of Experimental Surgery, University of Edinburgh.

EXPERIMENTAL

Adult stock mice of either sex were used in all experiments, and were well fed except when stated otherwise. The mice were killed by dislocating the vertebrae and the livers were then rapidly removed and cooled in crushed ice. Slices were cut, by the procedure of Cohen (1945), into chilled bicarbonate Ringer or phosphate Ringer solution (Krebs & Henseleit, 1932), in which the $MgSO_4$ was replaced by $MgCl_2$. The phosphate Ringer solution contained only one-third of the recommended amount of $CaCl_2$, to avoid precipitation of $Ca_3(PO_4)_2$. DL-Lactic, D-gluconic, D-saccharic

(acid K salt), succinic, fumaric, L-malic, L-glutamic and citric acids were neutralized before use with NaOH, using the glass electrode. D-Glucurone was treated with the equivalent quantity of $NaHCO_3$ solution. Pyruvic acid was added as the crystalline Na salt. The slices were shaken for 1 hr. at 38° in presence of $2{\cdot}3 \times 10^{-4}$ M-*o*-aminophenol and 0·001 M-ascorbic acid, the gas phase being 5% CO_2 in O_2 for bicarbonate Ringer solution, or pure O_2 for phosphate Ringer solution. After removal of the slices for dry-weight determination, protein was precipitated by a trichloroacetic acid-phosphate buffer mixture at pH 2·25, and the glucuronide diazotized and coupled with *N*-1-naphthylethylenediamine (Levvy & Storey, 1949). The turbidity sometimes encountered after adding the coupling reagent was removed by centrifuging for a short period before reading the extinction in a Spekker absorptiometer.

All results are the mean of four (or, in a few cases, of three) determinations, and are expressed as μg. *o*-aminophenol (OAP) conjugated/mg. dry wt. liver/hr.

O_2 uptakes were measured in Warburg manometers, using the above phosphate Ringer solution in pure O_2 at 38°. Each flask contained 2·2 ml. medium, and 0·2 ml. 20% (w/v) KOH with filter-paper rolls in the centre wells. Flasks were kept on ice until immediately before connecting to the manometers. After 10–15 min. for temperature equilibration, readings were commenced and continued for 70–90 min. The slices were then removed, washed in distilled water and dry weights determined after 2 hr. at 110°. Duplicate manometers were used in all experiments.

RESULTS

Attempts to stimulate synthesis

With liver slices from normal guinea pigs, Lipschitz & Bueding (1939) found that glucuronide formation was doubled in presence of lactate, and that when animals fasted for 20 hr. were used, increases of several hundred per cent were obtained with lactate and pyruvate. In the present experiments, even when the animals were fasted for very long periods, no significant increase in glucuronide synthesis could be demonstrated (Table 1). Similar results were obtained using phosphate Ringer solution. In another series of experiments, stimulation by lactate was not demonstrable by increasing the demand upon the glucuronide-synthesizing system, either by raising the concentration of *o*-aminophenol, or by adding another substance (borneol) known to form a glucuronide.

Table 1. *Influence of lactate and pyruvate on glucuronide synthesis by liver slices*

(Incubation 1 hr. (Exp. 5, 2 hr.) in bicarbonate Ringer solution; $2{\cdot}3 \times 10^{-4}$ M-OAP, 0·001 M-ascorbic acid.)

Exp. no.	Animal	Length of fast (hr.)	OAP conjugated/mg. dry wt. liver/hr. (μg.) Control	Lactate (0·02 M)	Pyruvate (0·02 M)
1	Mouse	—	0·68	0·70	—
2	Mouse	—	0·63	0·62	0·64
3	Mouse	—	0·93	0·92	—
4	Mouse	—	1·19	1·12	—
5	Mouse	15	0·51	0·50	—
6	Mouse	24	1·11	1·03	—
7	Mouse	44	0·63	0·62	0·64
8	Rat	24	0·61	0·70	—
9	Guinea pig	24	0·81	0·86	0·75
10	Guinea pig	24	1·21	0·99	0·99

It is evident that glucuronide synthesis can continue at its maximum rate even when carbohydrate stores in the liver are severely depleted. Under such conditions Lipschitz & Bueding (1939) working with guinea pigs, Meyerhof, Lohmann & Meier (1925) and Meyerhof & Lohmann (1926) using rats, obtained elevations of the $-Q_{O_2}$ up to 100% in some instances, when lactate was added to respiring liver slices from starved animals. Similar results have been observed during the present investigation (Table 2). In fully fed animals, lactate appears to have little or no effect on the respiration of liver slices. Although, as will be shown later, the synthesis does not take place under anaerobic conditions, these findings suggest that it is not appreciably influenced by a considerable depression in respiration.

Table 2. *Influence of* DL-*lactate on respiration of fasted mouse-liver slices*

(Mice fasted 28 hr. Phosphate Ringer solution, pH 7·35. Lactate present during equilibration.)

Exp. no.	$-Q_{O_2}$ (μl./mg. dry wt./hr.) Control	DL-Lactate (0·02 M)
1	5·0	8·1
2	7·8	13·8

Inhibition of synthesis by glucuronate

Whereas Lipschitz & Bueding (1939) used 0·02 M solutions of lactate and other substances in demonstrating stimulation of glucuronide synthesis, their negative results with glucuronic acid were obtained with a single concentration of 0·005 M, and it therefore seemed desirable to test this and related compounds at higher concentrations. In Table 3 is shown the effect of glucuronate, gluconate and saccharate, all in presence of 0·02 M-lactate, upon the synthesis, as compared with 0·02 M-lactate as control. Further results in the same series of experiments with a number of other compounds are shown in Table 4. The numbering of the experiments is the same for both tables. Of all the compounds tested, glucuronate is by far the most active, inhibition being frequently almost complete at a concentration of 0·02 M. Gluconate and saccharate, which are related structurally to glucuronate, also show considerable inhibition. Although at the higher concentration succinate and fumarate display activity almost equal to that of the first two named com-

Table 3. *Inhibition of glucuronide synthesis in mouse-liver slices by glucuronate and related substances*

(Incubated 1 hr. in bicarbonate Ringer solution; 0·02 M-lactate, $2·3 \times 10^{-4}$ M-OAP, 0·001 M-ascorbic acid. Well fed mice.)

		OAP conjugated/mg. dry wt. liver/hr. (μg.)													
		Glucuronate						Gluconate				Saccharate			
Exp. no.	Control	0·02 M	Inhibition (%)	0·01 M	Inhibition (%)	0·005 M	Inhibition (%)	0·02 M	Inhibition (%)	0·01 M	Inhibition (%)	0·02 M	Inhibition (%)	0·01 M	Inhibition (%)
1	0·54	0·12	78	—	—	0·41	24	—	—	—	—	0·26	52	0·45	17
2	0·44	0·04	91	0·18	59	—	—	—	—	—	—	—	—	—	—
3	0·51	0·01	98	0·07	86	—	—	0·28	45	—	—	0·32	37	—	—
4	0·68	0·14	79	0·39	43	—	—	—	—	—	—	—	—	—	—
5	0·77	—	—	—	—	—	—	0·39	49	—	—	0·50	35	—	—
6	0·54	0·06	89	0·37	31	0·42	22	0·33	39	—	—	—	—	0·42	22
7	0·76	—	—	0·47	38	0·69	9	—	—	—	—	—	—	—	—
8	0·60	0·17	72	—	—	—	—	—	—	—	—	—	—	—	—
9	1·04	0·15	86	—	—	—	—	0·65	38	—	—	0·70	33	—	—
10	1·16	0·40*	67	—	—	—	—	—	—	—	—	—	—	—	—
11	0·73	—	—	—	—	—	—	—	—	—	—	0·55	25	0·53	27
12	0·68	0·07	90	0·30	56	—	—	0·50	26	—	—	—	—	—	—
13	0·62	—	—	—	—	—	—	0·21	66	—	—	—	—	0·32	48
14	0·59	—	—	—	—	—	—	—	—	—	—	—	—	0·40	32
15	0·78	—	—	—	—	—	—	—	—	—	—	—	—	0·49	37
16	0·75	—	—	—	—	—	—	—	—	—	—	—	—	0·65	13
17	0·62	—	—	—	—	—	—	—	—	—	—	0·40	35	0·50	19
18†	0·54	—	—	—	—	—	—	0·29	46	—	—	0·25	54	—	—
19	0·63	—	—	—	—	—	—	0·39	38	0·52	17	0·26	59	0·53	16
20	0·61	—	—	—	—	—	—	0·32	48	0·56	8	—	—	0·36	41
21	0·43	—	—	—	—	—	—	0·15	65	0·25	42	—	—	0·14	67
22	0·30	—	—	0·06	80	—	—	—	—	0·19	37	—	—	0·23	23
23†	0·56	0·01	98	—	—	—	—	—	—	—	—	—	—	—	—
24†	0·80	—	—	—	—	—	—	—	—	—	—	0·49	39	—	—
Mean			85		56		18		46		26		41		30

* 0·36 in absence of lactate.

† Lactate not present in medium.

pounds, they (with malate) appear to be much less inhibitory at 0·01 M, and it seems possible that their mode of action, like that of lactate, pyruvate and glucose, may be different from that of the three compounds first mentioned. The results were essentially the same when lactate was omitted from the bicarbonate Ringer solution, and also when phosphate Ringer solution was employed, although measurement of inhibition was difficult owing to the low rate of synthesis in the controls in this medium (see p. 218).

The possibility that glucuronate reduced glucuronide synthesis by depression of the respiration of the liver slices was investigated by comparing slices from well fed mice, respiring in presence of 0·02 M-lactate, with others to which glucuronate was added at the beginning of the equilibration period. Since it was found that *o*-aminophenol itself, even in presence of lactate, caused a slight falling off in respiration after 40–50 min., experiments were performed with and without *o*-aminophenol and ascorbic acid, but the results were, in general, the same. Two typical experiments are shown in Fig. 1. Frequently, there appeared to be a slight initial stimulation of respiration by glucuronate lasting about 30 min. The oxygen uptakes with glucuronate showed considerable variation, even in the same experiment, a slight stimulation or a slight inhibition being observed at different times. It seems that any inhibition of the respiration of liver slices under the above conditions by 0·02 M-glucuronate is small. It certainly could not account for the inhibition of glucuronide synthesis since prolonged starvation, which depresses the respiration of liver slices considerably, has already been shown not to affect the synthesis. Saccharate, in a concentration of 0·014 M, was found by Karunairatnam & Levvy (1949) to have no effect on the respiration of mouse-liver slices.

Influence of oxygen tension

In agreement with Lipschitz & Bueding (1939), anaerobic conditions have been found to inhibit synthesis almost completely (Table 5). The gases used were taken directly from cylinders without purification. For experiments with air, phosphate Ringer solution was employed to simplify the technique.

It is evident that glucuronide synthesis requires the continuance of oxidative processes as a means of supplying energy. Decreasing the oxygen tension to that in air has no depressant effect on the synthesis,

Table 4. *Influence of various monocarboxylic and dicarboxylic acids and of glucose upon glucuronide synthesis by mouse-liver slices*

(Incubated 1 hr. in bicarbonate Ringer solution; 0·02 M-lactate, $2·3 \times 10^{-4}$ M-OAP, 0·001 M-ascorbic acid. Well fed mice.)

		OAP conjugated/mg. dry wt. liver/hr. (μg.)											
		Succinate				Fumarate				Lactate		Glucose	
Exp. no.	Control	0·02 M	Inhibition (%)	0·01 M	Inhibition (%)	0·02 M	Inhibition (%)	0·01 M	Inhibition (%)	0·02 M	Inhibition (%)	0·02 M	Inhibition (%)
4	0·68	—	—	—	—	—	—	—	—	0·56	18	—	—
12	0·68	—	—	—	—	—	—	—	—	0·64	6	—	—
14	0·59	0·45	24	0·51	14	—	—	—	—	0·56	5	—	—
15	0·78	—	—	0·58	26	—	—	0·56	28	—	—	—	—
16	0·75	—	—	0·75	0	—	—	0·75	0	—	—	—	—
25	0·65	—	—	—	—	—	—	—	—	0·56	14	—	—
26	0·62	0·29	53	0·42	32	—	—	—	—	—	—	0·52	16
27*	0·67	0·35	48	0·67	0	—	—	—	—	0·59	12	—	—
28	1·02	—	—	—	—	—	—	—	—	—	—	0·97	5
										Mean	11		11

		Malate		Pyruvate	
		0·01 M	Inhibition (%)	0·02 M	Inhibition (%)
8	0·60	—	—	0·47	22
18†	0·54	—	—	—	—
23†	0·56	—	—	—	—
24†	0·80	—	—	—	—
29	0·45	—	—	—	—
30	0·89	—	—	0·79	11
31	0·58	0·54	7	—	—
32	1·30	1·05	19	—	—
Mean			13		17

		Succinate				Fumarate				Lactate		Glucose	
Mean			38		13		38		9				

* Fasted 24 hr.

† Lactate not present in medium.

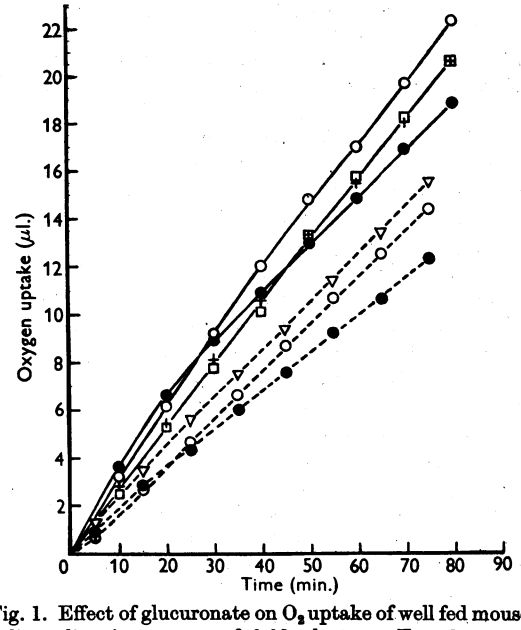

Fig. 1. Effect of glucuronate on O_2 uptake of well fed mouse-liver slices in presence of 0·02 M-lactate. Exp. 1: ○—○, +—+, controls; □—□, ●—●, 0·02 M-glucuronate. Exp. 2: ○ - - - - ○, control; ▽- - - -▽, ● - - - - ●, 0·02 M-glucuronate. Glucuronate added before equilibration.

at least for the low rates attainable in phosphate Ringer solution. This is to be expected as Laser (1937) showed that the respiration of rat-liver slices is depressed only to a small extent in air as compared with pure oxygen.

It might be argued that the inhibition of synthesis by glucuronate described in the previous section could be explained as an inhibition by excess substrate of β-glucuronidase, sufficient glucuronate being normally produced aerobically by the slices for synthesis to continue at its maximum rate. Such an explanation cannot, however, be correct since glucuronate fails to re-establish synthesis under anaerobic conditions when, on this line of thought, there would be no 'endogenous' production of glucuronate.

As would be expected (see preceding section), the respiratory inhibitor cyanide inhibits synthesis (Table 6), in agreement with Lipschitz & Bueding (1939), who obtained complete suppression of glucuronide formation with 0·001 M-potassium cyanide. According to Van Heyningen (1935), this concentration of cyanide inhibits the respiration of liver slices by 75–85 %.

De Meio & Arnolt (1944) have suggested that glucuronide synthesis might take place in the body by a reversal of hydrolysis coupled with an energy-

Table 5. *Influence of oxygen tension on glucuronide synthesis by mouse-liver slices*

($2{\cdot}3 \times 10^{-4}$ M-OAP, 0·001 M-ascorbic acid. Incubation for 2 hr. Yellow phosphorus in centre well to absorb O_2 in Exps. 1 and 2.)

Exp. no.	Medium	Gas phase	Glucuronate (M)	OAP conjugated/ mg. dry wt. liver/hr. (μg.)
1	Bicarbonate Ringer solution + 0·02 M-lactate	5% CO_2 in O_2	—	0·44
		5% CO_2 in N_2	—	0·00
		5% CO_2 in N_2	0·004	0·00
2	Bicarbonate Ringer solution + 0·02 M-glucose	5% CO_2 in O_2	—	1·35
		5% CO_2 in N_2	—	0·03
		5% CO_2 in N_2	0·004	0·03
		5% CO_2 in N_2	0·001	0·03
3	Phosphate Ringer solution + 0·02 M-lactate	100% O_2	—	0·12
		Air	—	0·12
		100% N_2	—	0·05
4	Phosphate Ringer solution + 0·02 M-lactate	100% O_2	—	0·09
		Air	—	0·09
		100% N_2	—	0·02

Table 6. *Inhibition of glucuronide synthesis in mouse-liver slices by cyanide*

(Bicarbonate Ringer solution; 0·02 M-lactate, $2{\cdot}3 \times 10^{-4}$ M-OAP, 0·001 M-ascorbic acid. Incubation 1 hr.)

Exp. no.	KCN (M)	OAP conjugated/ mg. dry wt. liver/hr. (μg.)	Inhibition (%)
1	—	0·81	—
	0·001	0·05	94
2	—	0·53	—
	0·001	0·03	94
	0·0005	0·06	89

Table 7. *Stability of o-aminophenylglucuronide in presence of normal or cyanide-poisoned mouse-liver slices*

(Bicarbonate Ringer solution; 0·02 M-lactate, 0·001 M-ascorbic acid.)

Exp. no.	Incubation (min.)	Cyanide (M)	*o*-Aminophenylglucuronide Added (μg.)	*o*-Aminophenylglucuronide Found (μg.)
1	120	—	11·1	12·1
		0·001	11·1	11·9
		0·0005	11·1	11·8
2	150	—	11·7	12·0
		0·0027	11·7	12·0
		0·0027*	11·7	12·1

* Incubated 20 min. before adding cyanide.

yielding mechanism. Blocking of the respiratory process by cyanide should then result in hydrolysis (cf. Borsook & Dubnoff, 1947). This was investigated by shaking liver slices with *o*-aminophenylglucuronide in presence of varying concentrations of potassium cyanide (Table 7).

It will be seen that no hydrolysis occurred either with the control slices, or with those poisoned with cyanide. This experiment must be interpreted cautiously since permeability may possibly be a factor. However, the result was the same in Exp. 2, even when time was allowed for penetration of the liver cells by the glucuronide (cf. Friedenwald & Becker, 1948) before poisoning with cyanide.

Inhibition by azide

It has been known for some years that azide is able to prevent a variety of assimilatory and related processes. Although the original observations of Keilin (1937) that azide strongly inhibited both the respiration of yeast and the cytochrome oxidase system of heart might explain the effect under aerobic conditions, they are obviously insufficient to explain the influence upon anaerobic metabolism (Winzler, Burk & du Vigneaud, 1944; Brockmann & Stier, 1947; Spiegelman, Kamen & Sussman, 1948). It is now evident that, at concentrations which do not inhibit respiration, azide can still affect aerobic processes (Winzler, 1940; Hotchkiss, 1944; Giese, 1945; Clifton, 1946). All these findings can be explained by the demonstration by Loomis & Lipmann (1948, 1949) that azide interferes with the generation of high-energy phosphate bonds. Since it is generally accepted that such bonds form the primary source of energy for animal tissues in both aerobic and anaerobic metabolism, it appeared of interest to study glucuronide synthesis in presence of concentrations of azide which would not affect respiratory activity.

Laser (1942) observed that 0·002 M-sodium azide inhibited the respiration of mouse-kidney slices by 40–60 % at pH 7·3, and 60–80 % at pH 6·8. Such a variation with pH, first demonstrated by Keilin (1937) for yeast, was also shown to hold for rat brain and muscle by Hollinger, Fuhrman, Lewis & Field (1949). Since no record of the influence of azide on liver respiration could be found in the literature, a study has now been made using the livers of fully

fed mice. The phosphate Ringer solution contained 0·02 M-lactate, but no *o*-aminophenol or ascorbic acid, and the pH was 7·3–7·4. The results were the same when the azide was added to the slices either before the equilibration period, or from the side bulb during the course of the experiment. Over the first hour of observation, in three experiments with 0·0005 M-azide and seven with 0·001 M-azide, no inhibition was observed. In two cases at the higher concentration, a slight falling off in the respiration became evident after 1 hr. 0·002 M-Azide showed a small inhibition in two experiments.

Since 1×10^{-4} M-DNP was found to increase the respiration of rat-liver slices 40 % (Franz, Zamecnik, Reese & Stephenson, 1948), this concentration was used to study the influence of DNP on glucuronide synthesis, and, as shown in Table 9, it causes almost complete inhibition. However, in view of the chemical relationship between *o*-aminophenol and DNP, it became necessary to consider whether this effect is really due to inhibition of high-energy phosphate formation, or whether the two compounds are merely competing for the enzyme system concerned in glucuronide synthesis. Accordingly, *o*-nitrophenol and 2-amino-4-nitrophenol were studied for comparison, and Table 9 shows that whilst the former is almost entirely non-inhibitory, the latter, though it depresses glucuronide formation, is much less active than DNP. When *o*-nitrophenol is fed to rabbits, it is conjugated to about 7 % as sulphate (Williams, 1938), and to a considerably greater extent as a glucuronide (Prof. R. T. Williams, private communication). It is the least toxic of the mononitrophenols, and Clowes & Krahl (1937) showed that it had no effect on either respiration or cell division in *Arbacia*. Although Heymans & Casier (1935) found that 2-amino-4-nitrophenol stimulated the metabolic rate in dogs and pigeons, the effect was only about 14 % of that caused by DNP; and Judah & Williams-Ashman (1949) were unable to detect any significant lowering of the P/O ratio in kidney

Table 8. *Inhibition of glucuronide synthesis in mouse-liver slices by sodium azide*

(Incubation 1 hr. in bicarbonate Ringer solution; 0·02 M-lactate, $2{\cdot}3 \times 10^{-4}$ M-OAP, 0·001 M-ascorbic acid.)

Exp. no.	Ringer solution pH	OAP conjugated/mg. dry wt. liver/hr. (μg.) Control	NaN_3 0·002 M	Inhibition (%)	NaN_3 0·001 M	Inhibition (%)	NaN_3 0·0005 M	Inhibition (%)
1	7·30	0·54	—	—	0·27	50	—	—
2	7·33	0·57	—	—	0·24	58	0·38	33
3	7·42	0·62	—	—	0·54	13	0·58	6
4	7·36	0·49	0·18	63	0·35	29	—	—
5	7·38	1·13	0·50	56	0·89	21	1·02	10
6	7·39	1·43	0·55	62	1·21	15	1·22	15
7*	7·32	0·48	—	—	0·20	58	—	—

* 0·02 M-glucose instead of lactate.

Table 9. *Effect of 2:4-dinitrophenol, o-nitrophenol and 2-amino-4-nitrophenol upon glucuronide synthesis by mouse-liver slices*

(Incubation 1 hr. in bicarbonate Ringer solution; $2{\cdot}3 \times 10^{-4}$ M-OAP, 0·001 M-ascorbic acid, 0·02 M-lactate.)

Exp. no.	OAP conjugated/mg. dry wt. liver/hr. (μg.) 1	2	3	4	5	6	7	8
Control	0·45	0·89	0·34	0·70	0·34	0·47	0·82	1·14
2:4-Dinitrophenol (1×10^{-4} M)	0·02*	0·02	0·06	0·22	0·07	0·06	0·06	0·15
Inhibition (%)	96	98	82	69	79	87	93	87
o-Nitrophenol (1×10^{-4} M)	—	—	—	0·75	0·31	—	0·71	—
2-Amino-4-nitrophenol (1×10^{-4} M)	—	—	—	—	—	0·15	0·34	0·48

* With $0{\cdot}2 \times 10^{-4}$ M-2:4-dinitrophenol, synthesis was 0·35 μg./mg./hr. (22 % inhibition).

In Table 8 is shown the influence of azide upon glucuronide synthesis, and it is evident that there is considerable interference at azide concentrations having no inhibitory effect on respiration.

Inhibition by 2:4-dinitrophenol

It is well known that 2:4-dinitrophenol (DNP) and a number of related nitrophenols are powerful stimulators of respiration. Like azide, they prevent a wide range of assimilatory processes (Martin & Field, 1934; Clowes & Krahl, 1937; Hotchkiss, 1944; Clifton, 1946; Spiegelman & Kamen, 1946), suggesting that the primary energy source of the cells is being interfered with. This is confirmed by the demonstration of Loomis & Lipmann (1948) that DNP reversibly uncouples the generation of high-energy phosphate bonds from oxidation.

homogenates at a concentration twice that of DNP which inhibited phosphate uptake 90%. As the latter authors (private communication) have pointed out, contamination of 2-amino-4-nitrophenol by small amounts of DNP might explain at least part of the observed effect of the former compound. Guerbet & Mayer (1932) showed that 2-amino-4-nitrophenol and 2-nitro-4-aminophenol were metabolic products of DNP, that they were sometimes present in urine in a combined form, and could be liberated by acid hydrolysis. This could explain the increased glucuronic acid excretion by dogs and rabbits after injection of DNP observed by Georgescu (1932). In the present work several attempts were made to detect glucuronide formation by liver slices in presence of DNP by prolonged ether extraction of the acidified Ringer solution followed by estimation of glucuronic acid according to Hanson, Mills & Williams (1944). The results were entirely negative, although it is possible that DNP-glucuronide, if formed, would not be ether-soluble. Incubation of 2-amino-4-nitrophenol and DNP with liver slices in absence of OAP gave products which yielded faint pink colours with the coupling reagents. The results (expressed in terms of μg. OAP conjugated/mg. dry wt./hr.) were as follows: at 1×10^{-4} M, 0·09 and 0·06 respectively; at 2×10^{-4} M, 0·12 and 0·00 respectively. As the colour in the case of 2-amino-4-nitrophenol was probably due to glucuronide formation, it seems likely that DNP was first reduced in the 2-position (cf. Greville & Stern, 1935). Since phenols generally form ester sulphates as well as glucuronides, it is of interest that DNP did not increase the excretion of ester sulphate when administered to a human subject (Tainter, Cutting & Hines, 1935). To summarize, the available evidence favours the view that DNP does not form a glucuronide. Its inhibitory effect on glucuronide synthesis must be due to some other, more highly specific, mechanism, and there can be little doubt, therefore, that inhibition of high-energy phosphate bond formation is the one concerned.

*The influence of bicarbonate**

At a fairly early stage of the work it was noticed that when phosphate Ringer solution was used the rate of glucuronide synthesis was always low. Further direct comparison of phosphate and bicarbonate Ringer solutions showed that synthesis in the bicarbonate medium was much greater than in the phosphate (Table 10). The increase was independent of the nutritional state of the animal, and lactate failed to cause any stimulation in phosphate, as in bicarbonate Ringer solution.

Following the technique of Warren (1944), various quantities of sodium bicarbonate solution were added to the oxygen-saturated phosphate Ringer solution, the liver slices were immediately added, the flasks filled with oxygen and stoppered. No provision was made for absorbing carbon dioxide. The bicarbonate concentrations in column 3 of Table 11 are calculated from the amount of bicarbonate solution added to the phosphate Ringer solution, and no account is taken of carbon dioxide lost to the gas

* Preliminary communication: see Storey (1949).

Table 10. *Comparison of glucuronide synthesis by mouse-liver slices in bicarbonate and phosphate Ringer solution*

(Incubation 1 hr.; $2{\cdot}3 \times 10^{-4}$ M-OAP, 0·001 M-ascorbic acid. Phosphate and bicarbonate Ringer solutions, with or without lactate. Mice fasted 24 hr., as indicated.)

	OAP conjugated/mg. dry wt. liver/hr. (μg.)				
Exp. no.	1	2	3	4	5
Phosphate Ringer solution	0·15	0·06	0·33	0·01	0·15
Bicarbonate Ringer solution	0·57	0·32	0·57	0·11	0·60
Lactate (0·02 M)	+	−	−	+	−
Fasted	−	+	−	+	+

Table 11. *Influence of bicarbonate upon glucuronide synthesis by mouse-liver slices*

(Incubation 1 hr.; $2{\cdot}3 \times 10^{-4}$ M-OAP, 0·001 M-ascorbic acid. Exp. 1, 0·02 M-lactate; Exp. 2, no lactate. Well fed mice. $NaHCO_3$ added to phosphate Ringer solution as shown.)

Ringer solution	Gas phase	Bicarbonate mM/l.	OAP conjugated/mg. dry wt. liver/hr. (μg.) Exp. 1	Exp. 2
Phosphate	O_2	—	0·34	0·14
Phosphate	O_2	1·5	0·38	—
Phosphate	O_2	2·7	—	0·35
Phosphate	O_2	6·0	0·47	—
Phosphate	O_2	7·8	—	0·54
Bicarbonate	95% O_2–5% CO_2	16·0*	0·53	0·62

* Assuming pH 7·30 (Umbreit, 1945).

phase during the incubation, or of respiratory carbon dioxide. It is evident, nevertheless, that quite small concentrations of bicarbonate stimulate glucuronide synthesis considerably. Since synthesis was the same at pH 7·4 as at pH 7·1 in phosphate Ringer solution, any possible pH effects on the addition of bicarbonate to this medium may be discounted.

Since it seemed unlikely that bicarbonate would favour the actual formation of the glycosidic linkage, these results suggested that carbon dioxide fixation played a part in glucuronide synthesis, and it became of interest to determine whether any of the known fixation reactions were concerned in the process. At present, only two are well established as occurring in animal tissues, namely, the addition of carbon dioxide to pyruvate to form a dicarboxylic acid (the Wood-Werkman reaction: Krebs & Eggleston, 1940; Evans & Slotin, 1941), and the carboxylation of α-ketoglutarate with the formation of a tricarboxylic acid, described by Ochoa (1945, 1948). According to recent work by Stern (1948), the second reaction is quantitatively the more important in mammalian liver.

If the step involving carbon dioxide fixation is the limiting one for glucuronide synthesis in phosphate buffer, the addition of the substance formed by fixation should stimulate the synthesis. The effect of certain components of the tricarboxylic acid cycle was therefore studied, using fasted mouse-liver slices in phosphate Ringer solution without added bicarbonate (Table 12). Glucose, galactose and lactate were also tested, for comparison. Succinate, malate and glutamate (in place of α-ketoglutarate) caused increases in some experiments, but the stimulation of synthesis was small, and the results in general were similar to those with glucose, galactose and lactate. This suggests that these small, irregular increases are due to the carbon dioxide produced as a result of the increased respiratory activity of the fasted slices in presence of the various substrates, rather than to any specific effects of these compounds on the synthesis. Little information can be gained as to the carboxylation of α-ketoglutarate, since citrate was a strong inhibitor of glucuronide synthesis.

Table 12. *Effect of dicarboxylic acids, lactate, citrate and carbohydrates on glucuronide synthesis by mouse-liver slices*

(Incubation 1 hr. in phosphate Ringer solution; $2·3 \times 10^{-4}$ M-OAP, 0·001 M-ascorbic acid. In Exps. 3, 4 and 9, animals were well fed; in remainder, fasted 16–24 hr.)

	OAP conjugated/mg. dry wt. liver/hr. (μg.)				
Exp. no.	Control	Succinate 0·01 M	Glucose 0·02 M	Galactose 0·02 M	Citrate 0·01 M
1	0·19	—	0·25	—	0·06*
2	0·16	—	0·15	0·18	—
3	0·21	—	0·23	0·17	—
4	0·33	0·39	—	—	0·28
5	0·11	0·12 0·11*	—	—	—
			Lactate 0·02 M	Malate 0·01 M	Glutamate 0·02 M
6	0·15	0·21	0·17	0·15	—
7	0·11	0·11	0·09	—	—
8	0·06	0·16	—	0·10	—
9	0·28	—	—	—	0·31
10	0·01	—	0·01	—	0·00

* 0·02 M-Succinate or citrate.

Inhibition by sulphate ion

Levvy & Storey (1949) stated that, when inorganic sulphate (0·0012 M) was present in the Ringer solution, 'the readings were, if anything, slightly lower than those obtained with slices from the same animal in sulphate-free Ringer'. It has now been found that sulphate ion causes considerable inhibition of glucuronide synthesis (Table 13).

The possibility that the inhibition was due to formation of an ester sulphate was investigated by estimating free *o*-aminophenol before and after acid hydrolysis.

o-Aminophenol was estimated by the colour reaction of Folin & Ciocalteu (1927). To 3 ml. of the phenol solution there was added 1 ml. of the Folin-Ciocalteu reagent diluted with an equal volume of water, followed by 4 ml. N-Na_2CO_3 solution. After mixing, the colour was developed at 37° for 20 min., and measured in a Spekker photoelectric absorptiometer, using the Ilford no. 602 blue filter. With a freshly prepared standard solution of resublimed *o*-aminophenol in 0·01 M-HCl, a linear relationship was obtained between the extinction and the amount of *o*-aminophenol in the range studied (0–60 μg.). Since both *o*-aminophenylglucuronide and ascorbic acid interfere in the colour development, it was necessary to extract the phenol from the Ringer solution. Preliminary experiments having showed that *o*-aminophenol was quantitatively extracted by ether from aqueous solution

above pH 5·2, M-acetate buffer, pH 5·8, was chosen, as it was considered desirable to extract below pH 7·0 to minimize oxidation of the free phenol. *o*-Aminophenylglucuronide and ascorbic acid were not extracted under these conditions.

General procedure. Mouse-liver slices were shaken in 5 ml. bicarbonate Ringer solution containing either $MgCl_2$ or $MgSO_4$, both 0·0048 M, for 1·5 hr. in 95% O_2–5% CO_2. The fluid (2 ml.) was then taken for determination of glucuronide synthesis in the usual manner.

Free phenol. The fluid (1 ml.) and M-acetate buffer (1 ml.), pH 5·8, were pipetted into the thimble of a liquid-liquid continuous extractor (Levvy, 1948), and extracted for 1·25 hr. with peroxide-free ether. The ether was then evaporated under diminished pressure, the residue taken up in 3 ml. water and the phenol estimated as described above.

Phenol after hydrolysis. To 1 ml. of the fluid in an extractor thimble, 0·1 ml. conc. HCl was added, and the tube immersed in a boiling-water bath for 10 min. After cooling, and the addition of 0·5 ml. 2 N-NaOH to neutralize excess acid and 1 ml. acetate buffer, pH 5·8, the procedure was as before.

In each of two further experiments, the inhibition of glucuronide synthesis (58 and 61 % respectively) produced by 0·0048 M-sulphate was not reversed by 0·004 M- or by 0·002 M-glucuronate. This is further evidence for the suggestion that free glucuronate does not participate in the system responsible for glucuronide synthesis.

Synthesis by tissues other than liver

Lipschitz & Bueding (1939) examined a considerable range of tissues for glucuronide-synthesizing ability, but with the exception of kidney all the results were negative. During the present work, in two experiments the following figures were found (results in terms of μg. OAP conjugated/mg. dry wt./hr.): liver, 0·79 and 0·61; kidney, 0·09 and 0·04, respectively. No synthetic ability was observed with spleen slices.

Table 13. *Inhibitory effect of sulphate ion upon glucuronide synthesis by mouse-liver slices*

(Incubation 1 hr. in bicarbonate Ringer solution; 0·02 M-lactate, $2·3 \times 10^{-4}$ M-OAP, 0·001 M-ascorbic acid, equimolar quantities of either $MgCl_2$ or $MgSO_4$.)

		0·0024 M-SO_4^{--}		0·0012 M-SO_4^{--}	
Exp. no.	Without SO_4^{--} (OAP conjugated/ mg. dry wt. liver/hr.) (μg.)	OAP conjugated/ mg. dry wt. liver/hr. (μg.)	Inhibition (%)	OAP conjugated/ mg. dry wt. liver/hr. (μg.)	Inhibition (%)
1	0·66	—	—	0·54	18
2	0·83	—	—	0·66	20
3	0·92	—	—	0·55	40
4	0·68	0·46	32	0·53	22
5	1·10	0·49	55	0·53	52

Table 14. *Production of glucuronide and of ester sulphate by mouse-liver slices with or without sulphate ion in the medium*

(Incubation 1·5 hr. in bicarbonate Ringer solution; 0·02 M-lactate, $2·3 \times 10^{-4}$ M-OAP, 0·001 M-ascorbic acid, equimolar quantities of either $MgCl_2$ or $MgSO_4$.)

Flask no.	1	2	3	4
SO_4^{--} (M)	—	—	0·0048	0·0048
Liver (mg.)	24·8	30·5	27·2	27·5
	OAP/mg. dry wt. liver/hr. (μg.)			
Conjugated as glucuronide (*A*)	0·90	0·81	0·38	0·37
Free phenol (*B*)	2·73	2·05	0·88	1·22
Phenol after hydrolysis (*C*)	3·42	2·74	3·86	3·47
Ester phenol (*C* − *B*)	0·69	0·69	2·98	2·25
Total phenol (*A* + *C*)	4·32	3·55	4·24	3·84

To make the different experiments comparable, all results are referred to 1 mg. dry-weight liver as basis (Table 14). They show clearly that depression of glucuronide formation in presence of inorganic sulphate is accompanied by a large increase in phenol extractable only after hydrolysis in N-hydrochloric acid at 100° for 10 min. Since *o*-aminophenylglucuronide and phenylglucuronide are unchanged by this treatment, whereas phenylsulphuric acid is completely hydrolysed (Levvy & Storey, 1949), it is safe to assume that the acid-labile substance present is indeed an ester sulphate.

DISCUSSION

Since Lipschitz & Bueding (1939) were able to demonstrate the stimulating action of certain three-carbon atom compounds in a very clear-cut manner, it is not evident why their results could not be repeated. Their starved animals showed only a very slight conjugation, whereas in the present work a comparison of Tables 1 and 2 suggests that there was no appreciable difference between the two groups of animals. Nevertheless, the rates of synthesis found

in the two laboratories are approximately of the same order of magnitude.

Conclusive evidence that the enzyme β-glucuronidase is not concerned in the synthesis of glucuronides in the body has been presented by Levvy, Kerr & Campbell (1948); Karunairatnam & Levvy (1949) and Karunairatnam, Kerr & Levvy (1949). The findings that cyanide-poisoned slices do not hydrolyse *o*-aminophenylglucuronide, and that glucuronate is actually an inhibitor of the synthesis, are in accord with this view. The failure of glucuronate to relieve the inhibition caused by anaerobiosis and by sulphate ion further suggests that free glucuronate does not take part at any point in the glucuronide-synthesizing enzyme system, irrespective of its nature.

In recent years, considerable advances have been made in our knowledge of the mechanism of the synthesis of glycosidic linkages, by Cori (1939) in his studies on glycogen, Hanes (1940) on starch and Kalckar (1947) on nucleosides. In each case the reaction involves an aldose-1-phosphate and a phosphorylase. With liver phosphorylase the synthesis of glycogen is strongly inhibited by free glucose (Cori & Cori, 1940), and the inhibition is competitive (Cori, Cori & Green, 1943). Mannose, galactose, maltose, fructose and glucose-6-phosphate have very little inhibitory effect. Campbell & Creasey (1949) reported that gluconic and ascorbic acids were without influence on muscle phosphorylase. It has been shown in the present paper that glucuronate is a powerful inhibitor of glucuronide synthesis, and that its activity in this respect is much greater than that of any of the other compounds tested. By analogy, a reasonable hypothesis for the synthesis of a glucuronide would then be the reaction of the aglycone with glucuronic acid-1-phosphate under the influence of a phosphorylase, rather than the tentative suggestion of Lipschitz & Bueding (1939) that condensation of the aglycone with a three-carbon compound took place.

Although bicarbonate undoubtedly stimulates glucuronide synthesis in high degree, suggesting that carbon dioxide fixation is involved, there is no definite evidence for the reaction mechanism. The results in Table 12 might be taken as an indication that carboxylation of pyruvate is not involved, since malate and succinate were unable to replace carbon dioxide. Since citrate was strongly inhibitory, little can be adduced as to whether carboxylation of α-ketoglutarate is concerned. The magnitude of the effect of bicarbonate in Table 10 is, however, comparable with that observed by Krebs & Eggleston (1940) for the synthesis of citrate, α-ketoglutarate, fumarate and malate from pyruvate in pigeon liver, and suggests that the action of carbon dioxide is a more or less direct one.

Although it is now well recognized that azide can inhibit assimilatory processes in micro-organisms, whilst leaving the respiratory system unimpaired, a similar effect does not appear to have been described for mammalian tissues. The present work suggests that azide may be useful in the latter field in those cases where the use of DNP might be undesirable, e.g. owing to interference with analytical procedures. Taken together, the inhibitory effect of azide and of DNP upon glucuronide synthesis provides strong evidence that this process depends on a supply of high-energy phosphate bonds. If the hypotheses suggested in the preceding paragraphs are correct, then this finding receives at least a partial explanation, since this source of energy would be essential, ultimately, for the formation of glucuronic acid-1-phosphate, and probably also for the fixation of carbon dioxide, which is an endergonic process (Ochoa, 1946).

It has been known for many years that certain substances, such as phenol, are conjugated in the body to form both ester sulphates and glucuronides. *o*-Aminophenol is similarly conjugated, to approximately the same degree by both routes, in the rabbit (Williams, 1938, 1943). The results now presented show that the inhibitory effect of sulphate ion upon glucuronide synthesis is the result of competition between the enzyme systems concerned in the two modes of conjugation for the *o*-aminophenol available. The mice used in these experiments resemble the Rosario strain of rats of De Meio & Arnolt (1944), in that there was considerable ester sulphate formation in sulphate-free Ringer solution. That an acid-labile sulphate ester of *o*-aminophenol is formed confirms the view that sulphate conjugation is not measured in the method of Levvy & Storey (1949) for studying glucuronide synthesis.

SUMMARY

1. The synthesis of glucuronides by mouse-liver slices in the presence of *o*-aminophenol has been studied with particular regard to its stimulation and inhibition by various reagents.

2. The synthesis of glucuronides by liver slices of fasted animals in presence of *o*-aminophenol could not be stimulated by lactate or pyruvate.

3. Glucuronate (0·02 M) inhibits the synthesis in high degree. Saccharate and gluconate are much less effective, whilst various monocarboxylic and dicarboxylic acids have still lower inhibitory action. The significance of these results is discussed.

4. It has been found, in agreement with previous workers, that anaerobiosis and cyanide abolish synthesis. Hydrolysis of *o*-aminophenylglucuronide by cyanide-poisoned slices could not be detected.

5. Azide, at concentrations which do not inhibit respiration, and 2:4-dinitrophenol, inhibit glucu-

ronide synthesis, suggesting that high-energy phosphate bonds are required as a source of energy.

6. Bicarbonate has a markedly stimulating effect on glucuronide synthesis, suggesting that fixation of carbon dioxide may be involved in the process.

7. Sulphate ion inhibits glucuronide synthesis by forming ester sulphate, and it is concluded that the glucuronide and ester sulphate syntheses are mutually competitive.

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