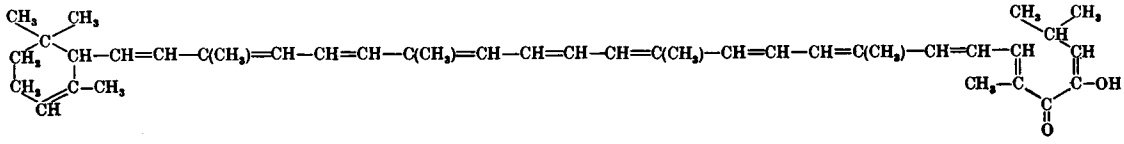
conjugated ketone groups, astacene and myxoxanthin, alteration of the absorption curve upon reduction of the ketone group has been shown for myxoxanthin only. The results obtained by different experimenters for astacene appear to be contradictory (Goodwin & Srisukh, 1949; Karrer & Würgler, 1943; Kuhn & Sörensen, 1938); however, one might assume that the later studies, employing more accurate instruments, are the more reliable ones.

By analogy with these ketonic carotenoids, it is likely that canthaxanthin is a carotenoid having a carbonyl group cross-conjugated in the system of double bonds. This idea is substantiated by the shape of the absorption curve of reduced canthaxanthin, the relative absorption maxima of the pigment and its reduction product, and the number of carbon-carbon double bonds found by catalytic hydrogenation. Our assumption that the oxygen



atoms are n the 3′ and 4′ positions, rather than distributed one to each end-group, is based on the tenuous evidence that the reduced pigment is not hypophasic in the usual partition tests as are all known polyhydroxy carotenoids having at least one hydroxyl group in each end-group.

Canthaxanthin appears to have the formula $C_{40}H_{54}O_2$ ($\pm$ 2H). Based on the foregoing arguments, the above structure is tentatively assigned to this compound.

Further experimental study is clearly required for more complete elucidation of the constitution of this carotenoid.

SUMMARY

1. The major carotenoid pigment obtained from an orange colour mutant of *Corynebacterium michiganense* is identical with canthaxanthin from the fungus *Cantharellus cinnabarinus*.

2. The compound appears to be a ketonic carotenoid, showing a single absorption maximum in the visible, with a probable formula $C_{40}H_{54}O_2$ ($\pm$ 2H) and a cross-conjugated carbonyl group. A tentative structure is suggested.

3. Reduction of canthaxanthin yields canthaxanthin-diol, a new carotenoid alcohol.

The writers acknowledge with thanks their indebtedness to Dr P. A. Ark for providing the culture and performing tests of virulence, to Dr H. J. Deuel, jun., for the provitamin A assay, to Dr A. J. Haagen-Smit for the catalytic hydrogenation, to Dr F. T. Haxo for a sample of fungal canthaxanthin and for confirmatory mixed chromatograms. The generous interest of Dr T. W. Goodwin, Dr F. T. Haxo, Dr C. B. van Niel and Dr L. Zechmeister during the study and preparation of this report is greatly appreciated.

REFERENCES

Amer. chem. Soc., Committee on Biochemical Nomenclature. (1946). *Chem. Engng News*, **24**, 1235.
Ark, P. A. (1951). *J. Bact.* **61**, 293.
Carr, F. H. & Price, E. A. (1926). *Biochem. J.* **20**, 497.
Goodwin, T. W. & Srisukh, S. (1949). *Biochem. J.* **45**, 263.
Haxo, F. (1950). *Bot. Gaz.* **112**, 228.
Karrer, P. & Jucker, E. (1950). *Carotenoids*, pp. 223, 225 and 353. New York: Elsevier Publ. Co., Inc.
Karrer, P. & Solmssen, U. (1935). *Helv. chim. acta*, **18**, 477.
Karrer, P. & Würgler, E. (1943). *Helv. chim. acta*, **26**, 116.
Kuhn, R. & Sörensen, N. A. (1938). *Ber. dtsch. chem. Ges.* **71**, 1879.
Saperstein, S., Starr, M. P. & Filfus, J. A. (1954). *J. gen. Microbiol.* **10**, 85–92.
Starr, M. P. & Saperstein, S. (1953). *Arch. Biochem. Biophys.* **43**, 157.

Uridine Compounds in Glucuronic Acid Metabolism

1. THE FORMATION OF GLUCURONIDES IN LIVER SUSPENSIONS

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Although much interest has been shown in recent years in the biochemistry of glucuronic acid, very few facts can be regarded as firmly established and virtually nothing is known of the mechanisms by which it is synthesized or incorporated into those compounds which contain it. We have accordingly extended earlier work on the synthesis of glucuronides (glucosiduronic acids) by liver slices (Storey, 1950) to broken-cell suspensions, in the hope that a study of this relatively simple process might yield some insight into such problems. In the course of this work we observed that glucuronide formation

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did not take place unless a boiled extract of liver was also added to the system (Dutton & Storey, 1951). This extract could not be replaced by any of a wide variety of metabolic intermediates and co-factors, and it was suspected that a hitherto unknown compound might be responsible. In subsequent work (Dutton & Storey, 1953) we have isolated, probably in a pure state, a nucleotide containing uridylic acid, labile phosphorus and glucuronic acid, which may have a structure somewhat analogous to the uridine diphosphate glucose of Leloir and co-workers (Caputto, Leloir, Cardini & Paladini, 1950), and which appears, under the conditions of incubation, to transfer its glucuronic acid to the *o*-aminophenol or menthol present.

The present paper describes the adaptation to suspensions of the method of Levvy & Storey (1949) for measuring glucuronide synthesis in tissue slices and the conditions necessary for inducing synthesis in such systems. Since the amounts of glucuronide synthesized were too small for direct isolation, indirect evidence is presented that the final colour measured was actually due to glucuronide formation. Crude preparations of the new factor were used to compare the synthesis of glucuronides in the suspension with that in slices and to make a brief examination of the enzyme system itself. In some instances, however, a relatively pure preparation of the factor was also employed. The isolation of the pure factor and observations on its chemical nature will be described in a further paper.

MATERIALS AND METHODS

Estimation of synthetic product. (*a*) o-*Aminophenol as substrate.* Since in the method of Levvy & Storey (1949) the final pH after protein precipitation must lie between 2·25 and 2·50, it was necessary to reinvestigate the precipitation conditions carefully as the amount of protein to be removed might be much larger than in slice experiments. Higher concentrations of phosphate buffer (2M) and trichloroacetate (1·25M), both solutions brought to pH 2·10 with NaOH, were essential to precipitate all protein, to overcome the buffering power of the medium and to bring the final pH to 2·25. Equal volumes of these two solutions were mixed before use, and 3·0 ml. added for every 3·0 ml. of incubation mixture. After centrifuging, 4·0 ml. of the supernatant were taken for diazotization and coupling with *N*-1-naphthylethylenediamine (Levvy & Storey, 1949).

(*b*) (−)-*Menthol as substrate.* The glucuronide formed was estimated by the naphthoresorcinol reaction as modified by Dr J. Paul (private communication), wherein menthyl-glucuronide is extracted with ethyl acetate before measurement by the Tollens method. Under these conditions the glucuronic acid present in the factor itself (Dutton & Storey, 1953) did not interfere.

Preparation of suspension. Mice were generally used as the source of liver suspension. After the animal had been killed by a blow on the neck or by dislocation of the vertebrae, the liver was removed at once and cooled in ice. It was then ground in a Potter-Elvehjem glass homogenizer with ice-cold alkaline isotonic KCl as suspending medium (0·154M-KCl containing $3·2 \times 10^{-4}$M-$KHCO_3$ (Potter, 1948)). The usual concentration was 1 g. tissue in 9 ml. alkaline isotonic KCl ('10% suspension'), but on occasion higher concentrations were employed. Water was also satisfactory as a suspending medium, but gave slightly lower rates of synthesis.

Measurement of glucuronide synthesis. This was carried out by a modification of the method of Levvy & Storey (1949). Unless otherwise stated, each 25 ml. conical flask contained 0·2–0·3 ml. of 0·5M potassium phosphate buffer, pH 7·4, 0·2 ml. of a solution containing 6 mg. *o*-aminophenol and 50 mg. ascorbic acid in 25 ml. water (this solution could be kept indefinitely at −20°), 0·1 ml. of 0·3M-$MgCl_2$, 0·5 ml. suspension and any additional substrates and water to a total volume of 3·0 ml. The final *o*-aminophenol concentration was 0·0015% ($1·4 \times 10^{-4}$M), since the concentration of 0·0025% used for liver-slice experiments was found to be inhibitory with suspensions. The suspension was added last, with the flasks standing on ice. The gas phase was air, except where specifically mentioned. In some of the initial experiments the phosphate buffer was replaced by a mixture of 0·15 ml. of 0·4M-$KHCO_3$ and 0·15 ml. of 0·5M potassium phosphate at pH 7·4, with a gas phase of 95% O_2 and 5% CO_2. Incubation was at 37° in a Warburg bath, with shaking. Since the reproducibility of the results was much greater with suspensions than with slices, it was sufficient to carry out determinations in duplicate. The results are expressed as μg. *o*-aminophenol conjugated/wet wt. liver used, in the time of incubation (usually 30 min.). When (−)-menthol was employed as substrate, the final concentration was 0·1% (0·0064M).

Phosphatase activity. This was assayed by the method of King (1946), using diphenyl phosphate as substrate. The unit of activity is that amount of enzyme liberating 1 mg. phenol in 15 min. incubation (Abul-Fadl, King, Roche & Thoai, 1949).

Mouse kidney phosphatase. The 10% water suspension of mouse kidney was diluted to 0·1% for use.

Mouse intestinal phosphatase. A 10% water suspension was prepared from the upper 4–6 in. of mouse small intestine, which had been slit lengthwise and washed in 0·9% (w/v) NaCl.

Dog intestinal phosphatase. Prepared according to Abul-Fadl *et al.* (1949), as far as the end of the second acetone precipitation.

Calf intestinal phosphatase. This specimen (a gift from Dr H. Klenow) had been purified according to Schmidt & Thannhauser (1943) by $(NH_4)_2SO_4$ fractionation up to the beginning of the adsorption stages.

Preparations

Cytochrome c was prepared from horse heart by the method of Keilin & Hartree (1945).

Diphosphopyridine nucleotide (*DPN*) was prepared from yeast by the method of Williamson & Green (1940). Spectrophotometric assay by the method of LePage (1947) showed a DPN content of 50%.

Adenosine triphosphate (*ATP*). A commercial preparation of the Ca salt was converted into the K salt by treatment with potassium oxalate.

α-D-*Glucuronic acid* 1-*phosphate.* A synthetic specimen of the tripotassium salt (Marsh, 1952) was a gift from Dr C. A. Marsh.

β-D-Glucuronic acid 1-phosphate. The Ba salt (Touster & Reynolds, 1952) was a gift from Dr O. Touster. It was converted into the K salts by dissolving in dilute HCl, treating with K_2SO_4 and neutralizing to pH 7·4 with KOH.

Substrates and cofactors were converted where necessary into the K salts before addition to the reaction flasks, the pH being checked generally by the glass electrode, or by indicator paper if the volume of solution available was very small.

RESULTS

Interference from β-glucuronidase

It was initially necessary to find out whether any glucuronide that might be formed in the suspension system would be destroyed at pH 7·4 by the β-glucuronidase present. Known amounts of *o*-aminophenylglucuronide were added to the system containing suspension, buffer, magnesium ions and ascorbic acid, with the *o*-aminophenol omitted, but with the addition of varying concentrations of potassium saccharate as a β-glucuronidase inhibitor (Karunairatnam & Levvy, 1949). After incubation for an hour in absence of saccharate, only slight hydrolysis (5 %) had taken place, and all hydrolysis could be prevented by concentrations of 0·0001 M and upwards. This concentration of saccharate was therefore added as routine in the early experiments, but it was generally omitted subsequently.

Attempts to induce glucuronide synthesis in suspensions

When *o*-aminophenol, ascorbic acid and suspension in a bicarbonate-phosphate buffer to a total volume of 3 ml., and gassed with oxygen and carbon dioxide, were incubated for varying periods with or without saccharate, the only evidence for the formation of *o*-aminophenylglucuronide was the gradual development, after diazotization and coupling, of a very faint pink colour, which was absent from unincubated mixtures, from those with boiled suspension and from those without *o*-aminophenol. This colour will be subsequently referred to as the 'endogenous' level of synthesis. The amount of *o*-aminophenol conjugated was never more than 10–15 % of the activity shown by a corresponding weight of liver slices in bicarbonate Ringer (Storey, 1950).

Since the performance of synthetic reactions appears to require high-energy phosphate, the effect of ATP or a system known to generate ATP from adenosine 5′-phosphate by oxidative phosphorylation was studied. A large number of experiments was performed with systems containing magnesium ions, fumarate, α-keto acids, DPN, cytochrome *c* and adenosine 5′-phosphate in varying proportions and combinations, but an appreciable increase in the conjugation was not observed on any occasion. (Nicotinamide added to inhibit breakdown of DPN appeared to cause oxidation of the *o*-aminophenol with the formation of a brown product.) Magnesium itself caused some stimulation (see later), but the further addition of ATP was only slightly more effective. When, however, it was found that the production of the colour was not decreased under anaerobic conditions, it seemed possible that the suspension system lacked some specific factor or group of factors, rather than an energy source. A large number of possible cofactors and intermediates was accordingly examined, but, as reported elsewhere (Dutton & Storey, 1951) an appreciable effect was not found in any case. Many of these substances were also tested in the phosphorylating system described above, but an increase over the 'endogenous' synthesis was not obtained.

The effect of boiled liver extract

Although increasing the suspension concentration gave a distinct increase in the final 'endogenous' colour, precipitation difficulties limited the amount which it was practicable to add. It did, nevertheless, suggest that the suspension contained some factor which increased the conjugation process, and a boiled extract was accordingly prepared and added to the incubation mixture in the hope that this factor, if thermostable, would then be present in sufficient amount to increase the conjugation without incurring difficulties in protein precipitation.

Fresh guinea pig liver (12 g.) was disrupted in 10 ml. of 0·5 % potassium chloride, poured into 30 ml. boiling potassium chloride solution and boiled for 10 min. The mixture was cooled and centrifuged, giving a cloudy pale-green solution. This supernatant ('boiled extract') was added to the system in varying amounts. Suitable controls, either unincubated or with the *o*-aminophenol added after incubation, were set up to allow for interference from glycogen and substances in the extract and suspension giving pink colours immediately after diazotizing and coupling. The results (Table 1) showed that the amount of *o*-aminophenol conjugated had greatly increased, to

Table 1. *Effect of boiled liver extract on glucuronide synthesis by liver suspension system*

The digests contained 0·3 ml. phosphate:bicarbonate buffer, pH 7·4, 0·2 ml. *o*-aminophenol:ascorbic acid, 0·1 ml. 0·3 M-$MgCl_2$, 0·5 ml. 10 % mouse-liver suspension, extract and water to 3·0 ml. Incubation for 30 min.

Extract added (ml.)	*o*-Aminophenol conjugated (μg./50 mg. wet wt. liver)
0	0·9
0·3	5·6
0·5	7·0
1·0	8·1
With slices	9·5

a level comparable with that of slices in the same medium. If the *o*-aminophenol was added after incubation, or if the suspension itself was boiled, conjugation did not take place.

These experiments seemed to show that the boiled extract contained a factor which in the presence of the suspension and *o*-aminophenol resulted in the formation of *o*-aminophenylglucuronide, but experimental proof was needed that the product giving the colour was a glucuronide. Since the amount of the reaction product formed in these experiments was very small, actual isolation and chemical characterization were impracticable and it was necessary to adduce other evidence.

The nature of the synthetic product

Colour development. When large amounts of either the boiled extract or suspension were used, a pink coloration became visible immediately after adding the naphthylethylenediamine in the colour reaction, and thereafter did not increase further in intensity. This effect was also observed in the absence of *o*-aminophenol. Although interference was very slight at the levels of extract and suspension normally used, it was always allowed for by suitable controls. Probably *p*-aminobenzoic acid or some similar compound was responsible for this interference, which could be clearly distinguished from the very slow colour development given by the synthetic product and by authentic *o*-aminophenylglucuronide, which is complete only after 2 hr. at 25° and pH 2·25 (Levvy & Storey, 1949). A specimen of *o*-aminophenylsulphuric acid (kindly presented by Dr A. B. Roy) did not yield any colour under these conditions, showing clearly that ester sulphate was not being measured.

Tollens reaction. When (−)-menthol was used as substrate in the incubation with boiled extract, a strong purple coloration was obtained with naphthoresorcinol. The control without menthol was nearly colourless. This result, though not conclusive, made it highly probable that a glucuronide was being synthesized.

Action of β-glucuronidase. If the product were actually a glucuronide then it should be hydrolysable by β-glucuronidase, and this hydrolysis should be inhibited by saccharate (Karunairatnam & Levvy, 1949). The synthetic system with boiled extract was set up with only half the usual quantity of buffer. After incubation for 30 min., the pH was lowered by hydrochloric acid and buffered to pH 5·2 with 0·3 M citrate. After the addition of β-glucuronidase and saccharate (where necessary), hydrolysis was allowed to take place for 2·5 hr. Protein was then precipitated and the colorimetric estimation carried out as usual. In the control synthesis, β-glucuronidase was not added until after the second incubation. The experiment was repeated with an ox-spleen β-glucuronidase preparation kindly given by Dr R. I. Cox. The combined results are shown in Table 2, compared with a series in which known *o*-aminophenylglucuronide was added to a similar series of flasks, except that *o*-aminophenol was not present and the first incubation was omitted. It will be seen that the product synthesized by the addition of boiled liver extract to the suspension was identical with *o*-aminophenylglucuronide, both in its destruction by β-glucuronidase and in the inhibition of this process by saccharate. This result, taken together with those preceding, does not leave any reasonable doubt that glucuronide synthesis was being measured.

The preparation and properties of the crude active factor

The above experiments showed that boiled liver extract contained a factor which led to the formation of glucuronides when incubated with the suspension system. In this section the preparation of a crude but stable preparation of the factor and some observations on its chemical properties are recorded.

Table 2. *Hydrolysis of synthetic product by β-glucuronidase*

All flasks contained 0·1 ml. 0·5M phosphate buffer, pH 7·4, 0·5 ml. 10% suspension, 0·5 ml. boiled extract, 0·2 ml. *o*-aminophenol:ascorbic acid, 0·1 ml. 0·3M-$MgCl_2$, water to 2·0 ml. Incubation for 30 min., then 0·05 ml. N-HCl, 0·3 ml. 0·3M citrate buffer, pH 5·1, added; where indicated, also 0·4 ml. β-glucuronidase, 0·3 ml. 0·015M saccharate. Total volume 3·0 ml. Incubation for 2·5 hr. With known *o*-aminophenylglucuronide, *o*-aminophenol and first incubation omitted.

	o-Aminophenol conjugated (μg.)	
	Mouse liver glucuronidase	Ox spleen glucuronidase
Control synthesis	7·0	6·3
Synthetic product + β-glucuronidase	4·8	5·4
Synthetic product + β-glucuronidase + saccharate	6·8	6·0
Known *o*-aminophenylglucuronide	8·5	8·4
Known *o*-aminophenylglucuronide + β-glucuronidase	5·0	6·1
Known *o*-aminophenylglucuronide + β-glucuronidase + saccharate	7·5	8·2

Acetone precipitation. When a large excess of acetone was added to the boiled extract, a precipitate was thrown down which gave an active solution on redissolving in water. It could be stored for several weeks if kept *in vacuo* at 0° over P_2O_5 and it showed only a very small, immediate, pink colour in the colour reaction, but it contained much glycogen and inorganic salts. However, if the finely minced liver was extracted with a solution of trichloroacetic acid rather than with boiling potassium chloride solution, the glycogen could be precipitated with ethanol and the active factor by an excess of acetone.

Freshly excized rabbit liver (120 g.) was chilled in ice and disrupted in 50 ml. of ice-cold water in a high-speed blender. All subsequent operations were conducted at as low a temperature as possible. The mash was treated with 18 g. trichloroacetic acid (A.R.) in 40 ml. of water, gradually and with constant stirring. The precipitated protein became granular and the mixture could then be strained through muslin. To the cloudy yellow filtrate an equal volume of ethanol was added, the glycogen centrifuged off and the supernatant poured into 10 vol. of acetone. The precipitate was very hygroscopic and lost its activity rapidly if allowed to take up moisture. Neutralization of the solution after glycogen precipitation gave a much more stable preparation, and the resulting cream-coloured powder ('crude factor'), stored over P_2O_5 *in vacuo* at $-20°$, remained active for many months.

Stability. To obtain larger quantities of starting material the livers of the ox, sheep and pig were obtained at the slaughter-house as fresh as possible and were transported to the laboratory packed in ice. These sources, however, did not furnish an extract with detectable activity, whereas freshly removed livers from rats, mice and guinea pigs invariably did so. The suspicion that rapid destruction of the active factor took place after the death of the animal was confirmed by the observation that a delay of even a few minutes in removing the livers from the rabbits gave a greatly diminished yield.

Effect of acid and alkali. Solutions of the crude factor were made 0·1 N with respect to hydrochloric acid and potassium hydroxide, and the tubes placed in a boiling-water bath for varying periods. After being cooled and neutralized to pH 7·4, they were assayed for synthetic activity (Fig. 1).

Action of phosphatase. When it was found that the reaction took place anaerobically (see later), it was thought possible that the factor might be glucuronic acid 1-phosphate or some related compound, as suggested in earlier experiments (Storey, 1950); for although there was as yet no direct evidence that the factor was a phosphate ester, its rapid destruction in intact liver and by acid and alkali suggested that this might be the case. A study was therefore made of its stability towards alkaline phosphatase from different sources and of varying degrees of purity, the results being shown in Table 3. The crude alkaline phosphatase preparations destroyed the activity almost completely, whereas the more active partially purified enzymes were much less effective even though in higher concentration. These results might be explained if the factor were not a simple type of phosphate ester and the effectiveness of the crude preparations were due to other types of phosphatases.

The active factor was also attacked by an acid phosphatase preparation. With a 20 % mouse-liver suspension in 0·03 M acetate buffer at pH 5·0 for 30 min., 65 % of the activity was destroyed.

Distribution of the factor

The factor could not be demonstrated in boiled extracts of rat and mouse kidney, muscle and brain, or of dried wheat germ. Baker's yeast was also examined, but a boiled extract could not be used owing to the presence of large amounts of interfering material, presumably largely *p*-aminobenzoic acid. The yeast was therefore extracted with cold trichloroacetic acid, the starch precipitated with ethanol and the supernatant treated with 4 vol. of acetone. The product so obtained did not show any activity.

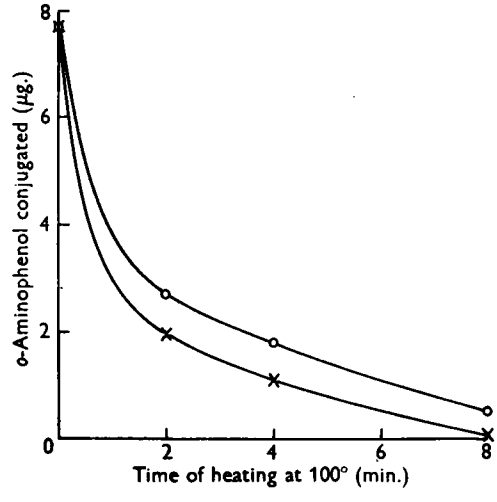

Fig. 1. Effect of acid and alkali on the activity of the crude factor. Crude factor (5 mg.) made 0·1 M with respect to HCl or KOH, heated in boiling-water bath for varying periods and neutralized to pH 7·4, then 0·2 ml. 0·5 M phosphate buffer, pH 7·4, 0·5 ml. 10 % suspension, 0·1 ml. 0·3 M-$MgCl_2$, 0·2 ml. *o*-aminophenol:ascorbic acid added. Total volume 3·0 ml. Incubation for 30 min. Values corrected for 'endogenous' synthesis. ×—×, 0·1 N-HCl; O—O, 0·1 N-KOH

Table 3. *Effect of alkaline phosphatase preparations on activity of factor*

The digests contained 1·0 ml. Na_2CO_3:$NaHCO_3$ buffer, pH 9·2, 0·5–1·0 ml. crude factor (10 mg./ml.), phosphatase and water to 2·1 ml. Incubation at 37°. N-HCl (0·1 ml.) added, heated at 100° for 2 min.; then 0·2 ml. 0·5M phosphate buffer, pH 7·4, 0·3 ml. 20 % mouse liver suspension, 0·2 ml. *o*-aminophenol:ascorbic acid, 0·1 ml. 0·3M-$MgCl_2$. Incubation at 37° for 30 min.

Enzyme preparation	0·1% Mouse kidney suspension	10% Mouse intestine suspension	Dog intestine	Calf intestine		
Activity (units/ml.)	2·3	0·08	40	500		
Units of enzyme added	0·23	0·008	7·2	1·0	1·0	10
Time of incubation (min.)	45	20	20	20	40	40
	o-Aminophenol conjugated (μg./60 mg. wet wt. liver)					
Untreated extract	4·1	3·3	5·5	8·1	5·3	4·1
Extract after treatment with phosphatase	0·9	1·7	4·3	6·0	3·3	1·9

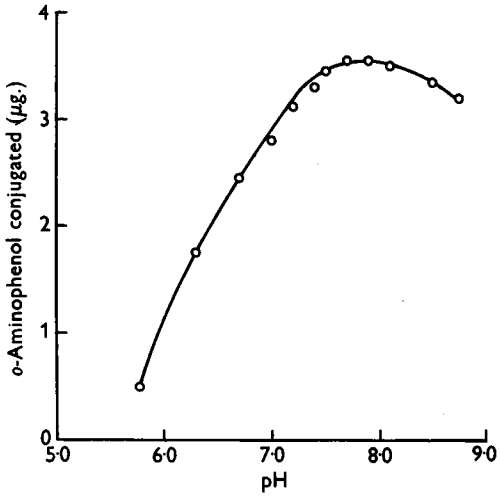

Fig. 2. Influence of pH on glucuronide synthesis, using Michaelis (1931) veronal buffer, with Na^+ replaced by K^+. Each flask contained 1·7 ml. buffer, 5 mg. crude factor and other additions as for Fig. 1. Total volume 3·0 ml. Incubation for 15 min.

Factors influencing the synthetic process

A study of the effects of various factors upon the synthetic reaction in suspensions was carried out to determine the optimum conditions of the process, and to enable it to be compared with glucuronide synthesis in liver slices. Although the crude factor was generally used in these experiments, in certain cases a much purer material obtained by barium: ethanol fractionation and chromatography on Dowex-1 anion-exchange resin (Dutton & Storey, 1953) was employed as well, but no essential difference between the two preparations was observed.

pH. Since the buffering power of phosphate was inadequate above pH 7·6, and caused precipitation with magnesium ion, the Michaelis (1931) veronal: acetate buffer was used, with potassium ion replacing sodium. The incubation period was only 15 min. and the pH was checked after incubation. From Fig. 2 it will be seen that there was a maximum at pH 7·6–7·9. In practice, however, pH 7·4 was chosen to avoid precipitation of magnesium.

Nitrogen and cyanide. In striking contrast with the suppression of glucuronide synthesis by slices under anaerobic conditions was the finding that suspensions incubated with either the crude or the partially purified factor in nitrogen did not show any depression when compared with those in air or pure oxygen. The 'endogenous' synthesis was likewise unaffected by anaerobic conditions. Cyanide (0·0004–0·01M) also did not depress the synthesis significantly when carried out under aerobic conditions, using either the crude or partially purified factor. It was therefore evident that the reaction was not dependent upon concurrent oxidative reactions.

Bicarbonate. Suspensions in isotonic potassium chloride were incubated either in 0·04M phosphate buffer with oxygen as the gas phase, or in a buffer containing 0·025M phosphate and 0·016M bicarbonate with oxygen containing 5 % carbon dioxide, the pH after equilibrating being 7·3. A difference between the amounts of *o*-aminophenylglucuronide synthesized in the two media could not be detected, even when cups containing 20 % potassium hydroxide were used to absorb traces of carbon dioxide in the case of the pure phosphate medium. These results were in marked contrast with the finding that liver slices may synthesize several times as much glucuronide in bicarbonate Ringer as in phosphate Ringer (Storey, 1950).

Sulphate. Storey (1950) found that 0·0012M sulphate caused considerable inhibition of glucuronide synthesis in liver slices. In the suspension system 0·005M sulphate did not show inhibition when compared with a control containing an equivalent amount of potassium ions added as the chloride.

Glucuronate and saccharate. D-Glucurone treated with the equivalent quantity of potassium bicarbonate solution showed some inhibitory effect (Table 4), though much less than in the slice experiments (Storey, 1950). A sample of 'potassium glucuronate' as supplied by Corn Products Ltd. (kindly given by Dr J. Paul) did not show any inhibition in the suspension system. A similar sample of 'sodium glucuronate', and glucurone neutralized with sodium hydroxide instead of sodium bicarbonate, did not show an inhibition with liver slices (private communication from Dr J. Paul, and unpublished observations). Saccharate, considerably less effective than glucuronate with slices, did not show any inhibition when added to the suspension up to a concentration of 0·015 M.

Table 4. *Influence of glucuronate on glucuronide synthesis*

The digests contained 0·5 mg. crude factor, 0·2 ml. 0·5 M phosphate buffer, pH 7·4, 0·5 ml. 10% suspension, 0·1 ml. 0·3 M-$MgCl_2$, 0·2 ml. *o*-aminophenol:ascorbic acid. Varying amounts of 0·03 M glucurone treated with the equivalent quantity of $KHCO_3$, water to 3·0 ml. Incubation for 20 min.

Glucuronate (M)	*o*-Aminophenol conjugated (μg.) Expt. 1	Expt. 2	Expt. 3
0	4·9	6·4	5·4
0·0005	4·5	—	—
0·001	4·6	—	—
0·005	3·9	5·3	—
0·01	—	4·7	—
0·02	—	4·1	3·4

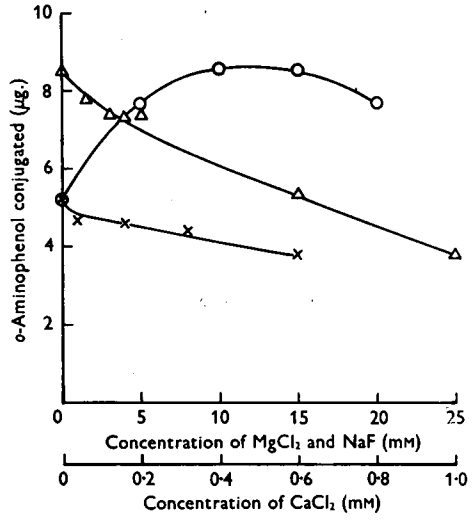

Fig. 3. Effect of ions upon glucuronide synthesis. Crude factor 5 mg., 0·5 M potassium phosphate buffer, pH 7·4, 0·2 ml., 10% liver suspension 0·5 ml., *o*-aminophenol: ascorbic acid 0·2 ml.; total volume 3 ml. Incubation for 30 min. ○—○, Varying amounts of 0·3 M-$MgCl_2$ added. △—△, 0·3 M-$MgCl_2$ 0·1 ml., 0·5 M potassium phosphate buffer pH 7·4 reduced to 0·1 ml., and varying amounts of 0·06 M-$CaCl_2$ added. ×—×, Varying amounts of 0·3 M-NaF added; in this experiment partially purified factor was used.

A solution of potassium hydrogen saccharate (0·05 M) was boiled under reflux for 1 hr. to cause formation of saccharic 1 → 4-lactone (Levvy, 1952) and diluted with 4 vol. of water. When added to the synthetic system at a final concentration of 0·0014 M (calculated as saccharate), no inhibition was observed. From the data of Levvy (1952), any decomposition of the lactone at pH 7·4 during the 30 min. incubation period would probably have been very small.

Magnesium. Magnesium ions exerted a favourable influence upon the course of the synthesis, as shown in Fig. 3, but since the enzyme system is a crude one it was not possible to decide whether it was essential. An added final concentration of 0·01 M was generally present in synthetic experiments.

Sodium and potassium. Although potassium ions were always used from the outset with the object of providing an environment as close to the intracellular as possible, synthesis in a system buffered with potassium phosphate was only slightly higher (about 8%) than when the sodium salt was used. The further addition of potassium or sodium ions to the sodium phosphate-buffered system did not have any apparent effect.

Calcium. Calcium ions, added as calcium chloride, showed a considerable inhibitory effect (Fig. 3).

Fluoride. Fluoride ions caused some inhibition, both with the resin-purified material (Fig. 3) and with the crude factor. Magnesium chloride was omitted from the medium in these experiments, but the inhibition might have been caused by the removal of magnesium ions introduced in the suspension.

Concentration of suspension. The rate of formation of *o*-aminophenylglucuronide was approximately proportional to the amount of suspension added (Fig. 4). The 'endogenous' synthesis is also shown, although the reaction in this case was generally complete within 10 min. The course of the reaction with different concentrations of suspension, using a purified preparation of the factor, is shown in Fig. 5, but similar curves were obtained with the crude factor.

Investigation of the enzyme system

Only preliminary studies were made on the enzyme system, for although a purified preparation would have been advantageous, it was soon realized that the system was labile and that its activity was readily destroyed by many of the usual purification procedures, such as ammonium sulphate fractionation and the preparation of acetone powders. The

stability of the enzyme system was investigated by keeping a 10 % mouse-liver suspension in alkaline isotonic potassium chloride for varying periods at 0°, at −20° or at 15°. After 1 hr. at 0° about 10 %, and after 24 hr. 60 % of the activity had been lost. Similar results were obtained at −20°. At 15° the decline in activity was rapid after approximately 0·5 hr.

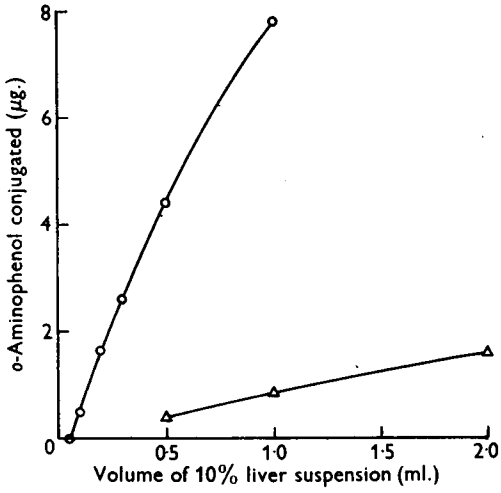

Fig. 4. Influence of concentration of liver suspension on glucuronide synthesis. △—△, Varying amounts of 10 % liver suspension, other additions as in Fig. 1. Incubation for 30 min. ('endogenous synthesis'). ○—○, As above, but with 5 mg. crude factor; values corrected for 'endogenous' synthesis.

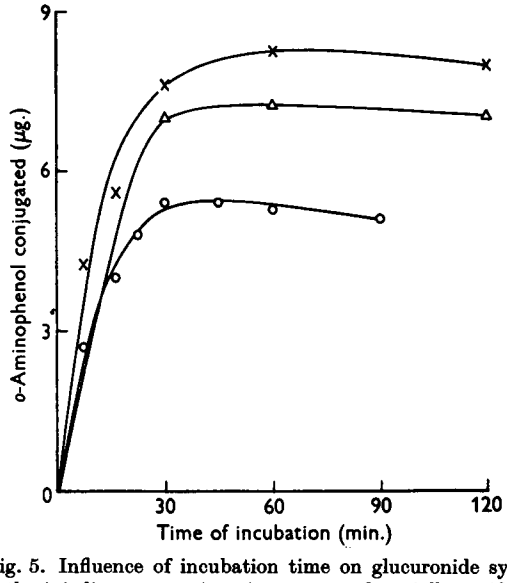

Fig. 5. Influence of incubation time on glucuronide synthesis in liver suspensions, in presence of partially purified factor. Other additions as in Fig. 1; ○—○, 0·5 ml. suspension; ×—×, 1·0 ml. suspension; △—△, 2·0 ml. suspension. All curves corrected for 'endogenous' synthesis.

After low-speed centrifuging of a 10 % suspension in alkaline isotonic potassium chloride for 5–10 min. at 1600 *g*, most of the activity was present in the supernatant, although the washed residue seemed to increase this activity slightly. The fractionation of rat-liver suspensions in 0·25 M sucrose according to Schneider & Hogeboom (1950) was therefore studied. The activity was absent from the low-speed residue (sedimented at 700 *g* for 10 min.) and from the high-speed supernatant (27 000 *g* for 1·5 hr.), but appeared to be connected with the larger (5000 *g* for 20 min.) and smaller cytoplasmic granules. Any conclusions from these experiments must be tentative, but it would appear that the enzyme in rat-liver suspensions is largely insoluble under these conditions.

Distribution. 10 and 20 % suspensions of mouse kidney, spleen, brain and small intestine were examined for the presence of the enzyme system both with and without added factor, but synthesis could not be detected.

DISCUSSION

Since the present glucuronide-synthesizing system is not affected by anaerobic conditions or by cyanide it evidently differs greatly from that in liver slices (Storey, 1950), which is dependent on concurrent oxidations. The system in suspensions is also not influenced by bicarbonate and sulphate ions. De Meio & Tkacz (1952) and Bernstein & McGilvery (1952) appear to have observed a process of glucuronide synthesis which requires a source of energy. That the process studied here was a simpler one seemed at first in accord with a previous suggestion that the final step in glucuronide synthesis might be the reaction between the aglycone and glucuronic acid 1-phosphate in presence of a phosphorylase (Storey, 1950). When the synthetic α- and β-D-glucuronic acid 1-phosphates became available, however, we were unable to find any synthesis of the glucuronide when either was added to the unfortified suspension at a concentration of 0·005 M. Levvy & Marsh (1952) had previously failed to observe synthesis with either isomer. The phosphatase experiments had also indicated that the active factor was probably not a simple type of phosphate ester. The net result of the reaction described in this paper appears to be the transfer of a molecule of glucuronic acid from the factor to the aglycone acceptor (Dutton & Storey, 1953). Evidence for this will be presented in a future publication.

Since it has been claimed that β-glucuronidase is concerned in glucuronide synthesis (for references see Levvy, 1953), it is of interest to inquire whether our results have any bearing on this view. Whereas β-glucuronidase has been demonstrated in nearly all the animal tissues studied and is particularly

abundant in the spleen, the system we have described is absent from that organ in the mouse and has so far been found only in the liver. This finding agrees with the results of tissue-slice experiments. The stability of β-glucuronidase to storage, to acetone precipitation and to ammonium sulphate fractionation is also in marked contrast with the above results. A purified spleen β-glucuronidase preparation (kindly given by Dr G. T. Mills) could not replace the liver suspension in the synthetic system. Although this result might be explicable if the system comprised more than one enzyme, we have found that saccharate and saccharic $1 \rightarrow 4$-lactone (the most powerful β-glucuronidase inhibitor known) do not interfere with the synthesis. Finally, Dr G. A. Levvy has very kindly studied the effect of a pure solution of the factor upon the hydrolysis of phenolphthalein glucuronide by mouse liver β-glucuronidase. A 4×10^{-5}M solution of the factor did not inhibit the hydrolysis of $12{\cdot}5 \times 10^{-5}$M or $6{\cdot}25 \times 10^{-5}$M substrate (within experimental error), and he concludes that the factor cannot be a substrate for β-glucuronidase. Taken together, the evidence appears to show conclusively that this enzyme is not concerned in the formation of glucuronides by the present system.

SUMMARY

1. Conditions are described for the study of glucuronide synthesis in liver suspensions.

2. Glucuronide synthesis could not be demonstrated unless a boiled extract of liver was also present. The reaction product has been shown to be a glucuronide.

3. The active factor obtained from liver by boiling or by trichloroacetic acid extraction was destroyed by acid and alkali and by phosphatase preparations.

4. The effect of pH and certain other conditions upon the synthesis has been studied.

5. The enzymic activity of the suspensions appeared to be located in the particulate material of the cell cytoplasm.

6. The synthesis in suspensions is compared with that in liver slices, and the possible relationship of β-glucuronidase to the first-named process discussed.

REFERENCES

Abul-Fadl, M. A. M., King, E. J., Roche, J. & Thoai, N-v. (1949). *Biochem. J.* **44**, 428.
Bernstein, S. & McGilvery, R. W. (1952). *J. biol. Chem.* **198**, 195.
Caputto, R., Leloir, L. F., Cardini, C. E. & Paladini, A. C. (1950). *J. biol. Chem.* **184**, 333.
De Meio, R. H. & Tkacz, L. (1952). *J. biol. Chem.* **195**, 175.
Dutton, G. J. & Storey, I. D. E. (1951). *Biochem. J.* **48**, xxix.
Dutton, G. J. & Storey, I. D. E. (1953). *Biochem. J.* **53**, xxxvii.
Karunairatnam, M. C. & Levvy, G. A. (1949). *Biochem. J.* **45**, 496.
Keilin, D. & Hartree, E. F. (1945). *Biochem. J.* **39**, 289.
King, E. J. (1946). *Micro-Analysis in Medical Biochemistry*, p. 53. London: Churchill.
LePage, G. A. (1947). *J. biol. Chem.* **168**, 623.
Levvy, G. A. (1952). *Biochem. J.* **52**, 464.
Levvy, G. A. (1953). *Brit. med. Bull.* **9**, 126.
Levvy, G. A. & Marsh, C. A. (1952). *Biochem. J.* **52**, 690.
Levvy, G. A. & Storey, I. D. E. (1949). *Biochem. J.* **44**, 295.
Marsh, C. A. (1952). *J. chem. Soc.* p. 1578.
Michaelis, L. (1931). *Biochem. Z.* **234**, 139.
Potter, V. R. (1948). *Meth. med. Res.* **1**, 317.
Schmidt, G. & Thannhauser, S. J. (1943). *J. biol. Chem.* **149**, 369.
Schneider, W. C. & Hogeboom, G. H. (1950). *J. biol. Chem.* **183**, 123.
Storey, I. D. E. (1950). *Biochem. J.* **47**, 212.
Touster, O. & Reynolds, V. H. (1952). *J. biol. Chem.* **197**, 863.
Williamson, S. & Green, D. E. (1940). *J. biol. Chem.* **135**, 345.

Studies in Immunochemistry

14. THE ISOLATION AND PROPERTIES OF SUBSTANCES OF HUMAN ORIGIN POSSESSING BLOOD-GROUP B SPECIFICITY

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The isolation and properties of human blood-group substances which possess A, H and Le^a specificity from ovarian cyst fluids are described by King & Morgan (1944), Morgan & Waddell (1945), Aminoff, Morgan & Watkins (1950) and Annison & Morgan (1952*a*, *b*), and certain of the materials were shown to be substantially homogeneous both physically and chemically. The present paper describes the isolation of substances having B specificity from the same source, and records their chemical, physical and serological properties. Earlier work in this field is reviewed by Morgan (1947), Bray & Stacey (1949) and Kabat (1949).

A number of useful procedures for the purification of blood-group substances derived from ovarian cyst fluids have already been described, and it has