physical and chemical methods used are compatible and that their combined application puts a tool of considerable power in the hands of biologists.

ACKNOWLEDGEMENTS

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SUMMARY

A study has been made of the cell walls of 15 species of marine algae. The cell walls were separated chemically into four fractions which were hydrolysed and analysed by paper chromatography for their constituent sugars. Before and after each chemical extraction samples were examined by X-ray diffraction analysis and electron microscopy.

The results indicate that the algae investigated may be divided into three groups on a basis of their cell wall structure. Only Group 1 (*Cladophora rupestris* and *Chaetomorpha melagonium*) shows any resemblance to the condition in higher plants by the presence of Cellulose I. The algae of Group 2 (comprising the remaining sp with the exception of *Porphyra*) are characterised by a crystalline microfibrillar fraction which is certainly not Cellulose I and hydrolyses to give a mixture of sugars always containing glucose and often xylose. *Porphyra* is quite unique and forms a third group in which mannose replaces glucose as the basic structural unit of the microfibrils.

The importance of the material presenting an amorphous appearance in the electron micrographs is stressed.

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URIDINE PYROPHOSPHOGLYCOSYL COMPOUNDS AND THE FORMATION OF GLUCURONIDES BY ISOLATED ENZYME SYSTEMS

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The isolation of uridine pyrophosphoglucuronic acid (UPPGA) from liver tissue by SMITH AND MILLS[1] and the demonstration that this compound acted as a glucuronic acid donor in the formation of glucuronides by liver homogenates opened the way to

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the elucidation of the pathway of glucuronide formation in the liver. The isolation of UPPGA[1] followed from the demonstration by DUTTON AND STOREY[2,3,4] of the presence in liver of a thermostable factor involved in glucuronide synthesis. In 1955 STOREY AND DUTTON[5] confirmed that this factor was UPPGA.

It was shown by SMITH AND MILLS[1] that the formation of UPPGA did not follow a pathway analogous to that for the formation of uridine pyrophosphoglucose (UPPG), the reaction for the latter being:

Uridine triphosphate (UTP) + glucose-1-phosphate $\rightleftharpoons$ UPPG + pyrophosphate

(MUNCH-PETERSEN, KALCKAR, CUTOLO AND SMITH[6]).

Although liver cell nuclei contain the enzyme uridyl transferase[1,7] UPPGA is not formed from UTP and either α- or β-glucuronic acid-1-phosphate in the presence of this enzyme nor in the presence of uridyl transferase from yeast[1].

In 1954 STROMINGER, KALCKAR, AXELROD AND MAXWELL[8] demonstrated the presence, in liver, of a DPN-linked dehydrogenase which converted UPPG into UPPGA and they later showed[9] that two molecules of DPN were reduced per molecule of UPPG oxidised to UPPGA. STROMINGER *et al.*[9] found this dehydrogenation reaction to be optimally active at pH 8.7 and irreversible.

These findings, coupled with the work of MUNCH-PETERSEN *et al.*[6,10,11] on the mechanism of formation of UPPG, and the demonstration of the presence of the enzyme uridyl transferase in liver cell nuclei[1,7] suggested that the route of formation of glucuronides from glycogen or glucose involves the formation of glucose-1-phosphate from glycogen or glucose-6-phosphate, the conversion of glucose-1-phosphate to UPPG, the oxidation of the latter compound to UPPGA and finally the transfer of glucuronic acid from UPPGA to an acceptor to form a glucuronide.

In the present paper, data is presented on the coupling of these various reactions in isolated enzyme systems which demonstrate the formation of glucuronides from glucose and glycogen.

A preliminary account of this work has already appeared (MILLS AND SMITH[12]).

MATERIALS AND METHODS

Adenosine triphosphate (ATP) (crystalline disodium salt), adenosine diphosphate (ADP), triphosphopyridine nucleotide (TPN) (80% purity), glucose-6-phosphate dehydrogenase (Practical, Type II) and hexokinase (Type II) were obtained from Sigma Chemical Company, St. Louis, Mo., U.S.A.

Glucose-1-phosphate (G-1-P), glucose-6-phosphate (G-6-P), diphosphopyridine nucleotide (DPN) (95% purity), reduced diphosphopyridine nucleotide (DPNH) (65% purity) and α-oxoglutarate were obtained from Boehringer and Soehne, Mannheim, W. Germany.

Uridine triphosphate (UTP) was obtained from Pabst Laboratories Milwaukee, Wisconsin, U.S.A.

UPPG was prepared from baker's yeast (Distillers Co. Ltd.) by the method of CAPUTTO, LELOIR, CARDINI AND PALADINI[13] and purified by ion exchange chromatography (CABIB, LELOIR AND CARDINI[14]). The preparation used was free from uridine pyrophosphogalactose (UPP galactose) (*cf.* MILLS, SMITH AND LOCHHEAD[15]).

Uridine pyrophosphate (UPP) was prepared from UPPG by hydrolysis at pH 2 for 15 minutes in a boiling water bath.

UPPG dehydrogenase was prepared from calf liver by the method of STROMINGER, MAXWELL, AXELROD AND KALCKAR[9]. The concentration of the enzyme preparation was adjusted so that 30 μl produced a ΔE_{340} of 0.100 per minute when assayed in the presence of 0.05 μmole UPPG, 0.5 μmole DPN and 0.1*M*-TRIS buffer pH 9.1 to 1 ml.

Zwischenferment was prepared from dried brewer's yeast (Kongens Bryghus, Copenhagen)

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by the method of LEPAGE AND MUELLER[16]. This preparation contains, in addition to glucose-6-phosphate dehydrogenase, the enzymes uridyl transferase and nucleoside diphosphokinase[1,10].

Uridyl transferase was prepared from dried brewer's yeast (Kongens Bryghus, Copenhagen) by the method of MUNCH-PETERSEN[11].

Glutamic dehydrogenase was prepared from an acetone powder of calf liver by the method of KORNBERG AND PRICER[17].

Phosphoglucomutase was prepared by the method of NAJJAR[18]. The preparation was taken as far as the "second heat filtrate".

Muscle phosphorylase was prepared by the method of GREEN AND CORI[19]. The preparation was taken as far as the dialysis against glycerophosphate and cysteine but was not crystallised.

o-Aminophenylglucuronide (*o*-APG) formation was measured by the method previously described[1].

RESULTS

The formation of UPPGA and o-APG from UPPG

The relationship between the formation of UPPGA from UPPG when assayed spectrophotometrically in the presence of UPPG dehydrogenase and DPN, and when assayed by *o*-APG formation in a two-step reaction is shown in Fig. 1.

It will be seen from Fig. 1 that in the presence of excess DPN there is an almost quantitative conversion of UPPG to UPPGA and that the overall formation of *o*-APG is approximately 85 %.

It was considered that, in the presence of a DPN regenerating system, much smaller amounts of DPN than was used in Fig. 1 would allow the conversion of considerable amounts of UPPG to UPPGA. The results of an experiment to test this hypothesis, using glutamic dehydrogenase with α-oxoglutarate and NH_3 as the DPN regenerating system, are presented in Fig. 2; the overall *o*-APG formation from UPPG is shown in this figure.

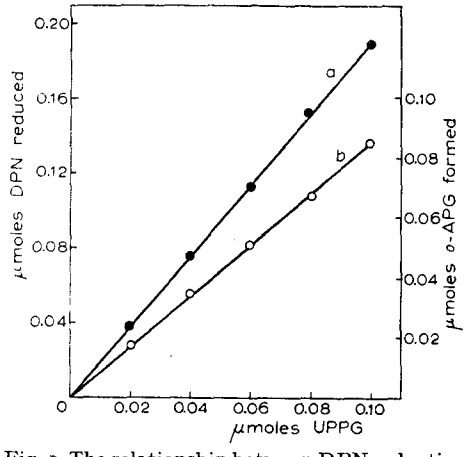

Fig. 1. The relationship between DPN reduction by UPPG dehydrogenase and UPPG and the formation of *o*-APG. Curve (a). Varying amounts of UPPG incubated with 1 μmole DPN, 30 μl UPPG dehydrogenase and 0.1 *M*-TRIS buffer pH 9.1 to 1.0 ml. Total ΔE_{340} measured against control run without UPPG. Curve (b). Incubation mixture from (1) heated for 30 seconds in boiling water bath, cooled, centrifuged, supernatant adjusted to pH 7.7, and UPPGA measured by *o*-APG formation.

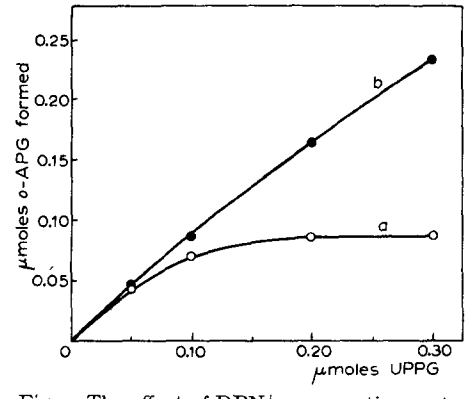

Fig. 2. The effect of DPN^+ regenerating system on the overall formation of *o*-APG from UPPG in the presence of limiting amounts of DPN. Curve (a). As for curve (b), Fig. 1, using 0.2 μmole DPN. Curve (b). As for curve (a), with the addition of 20 μl glutamic dehydrogenase, 2 μmoles α-oxoglutarate and 2 μmoles NH_4Cl.

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It will be seen from Fig. 2 that when 0.2 μmoles DPN is used in the absence of the DPN regenerating system, the *o*-APG formed reaches a maximum of 0.085 μmoles, the amount expected from the DPN present. However, in the presence of the DPN regenerating system, the *o*-APG formed is approximately proportional to the amount of UPPG used. The curve departs slightly from linearity at the higher concentrations of UPPG; this may be due either to a small breakdown of the UPPGA formed or to a suboptimal rate of reaction for the UPPG dehydrogenase. At the highest level of UPPG used, the *o*-APG formed represents a 77 % yield.

The effect of UTP and G-1-P concentrations on the rate of formation of UPPGA

The demonstration by MUNCH-PETERSEN *et al.*[6,10,11] that the mechanism of UPPG formation is by means of the reaction UTP + G-1-P ⇌ UPPG + P-P and the demonstration that the enzyme uridyl transferase is present in liver[1,7] strongly suggests that this reaction is operative in glucuronide formation and that UPPGA formation from UTP and G-1-P may be estimated by following DPN reduction in the presence of uridyl transferase and UPPG dehydrogenase.

The relative rates of formation of UPPGA, as measured by the ΔE_{340} of DPN reduction, from UPPG, from UTP and G-1-P and from UPP, ATP and G-1-P in the presence of the requisite enzyme systems are shown in Fig. 3. Fig. 4 shows the relationship between UPPGA formation and UTP concentration with a constant amount of

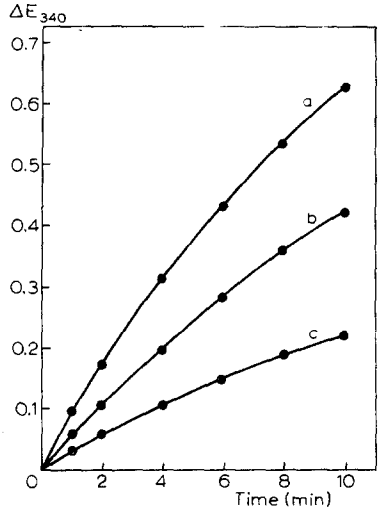

Fig. 3. The relative rates of formation of UPPGA (as measured by DPN reduction) from (a) UPPG, (b) UTP and G-1-P and (c) UPP, ATP and G-1-P. Curve (a). 0.1 μmole UPPG incubated with 0.5 μmole DPN, 30 μl UPPG dehydrogenase and 0.1*M*-TRIS buffer pH 8.5 to 1.0 ml. Curve (b) As curve (a) using 0.1 μmole UTP, 0.5 μmole G-1-P and 0.5 mg Zwischenferment in place of UPPG. Curve (c). As curve (a) using 0.1 μmole UPP,0.2 μmole ATP, 0.5 μmole G-1-P and 0.5 mg Zwischenferment in place of UPPG. Corrections made for controls run without UPPG, UTP or UPP.

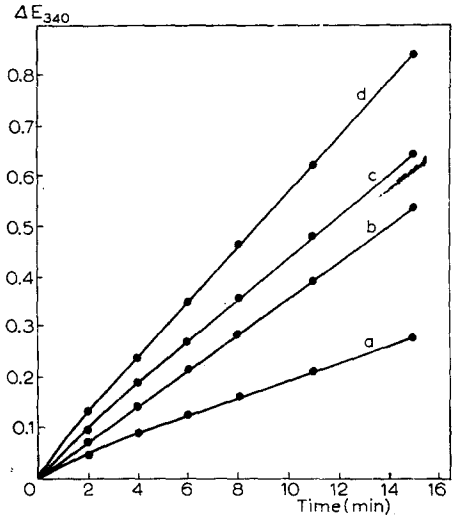

Fig. 4. The effect of UTP concentration on the rate of UPPGA formation. Incubation mixtures contained 1.0 μmole G-1-P, 1.0 μmole DPN, 30 μl UPPG dehydrogenase, 0.5 mg Zwischenferment, 5 μmoles $MgCl_2$, 0.1*M*-TRIS buffer to 1.0 ml and UTP as indicated. Curve (a) 0.05 μmole UTP. Curve (b) 0.1 μmole UTP. Curve (c) 0.2 μmole UTP. Curve (d). 0.5 μmole UTP. Corrections made for controls run without UTP.

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G-1-P. The relative rates of UPPGA formation from G-1-P, G-6-P and glucose in the presence of UTP and the requisite enzyme systems are presented in Fig. 5.

From the results presented in Fig. 3, 4 and 5 it is clear that UPPGA can be formed from UTP and either G-1-P, G-6-P or glucose using the requisite enzyme systems and that the rate of reaction depends upon the UTP concentration. In addition it will be seen from Fig. 3 that UTP can be replaced by UPP and ATP in the presence of nucleoside diphosphokinase.

In an experiment similar to that recorded in Figure 4 it was found that the rate of UPPGA formation depended upon the G-1-P concentration when the UTP concentration was constant.

The lower rate of formation of UPPGA from G-6-P and from glucose and ATP is probably a reflection of the position of the phosphoglucomutase equilibrium which is very much in favour of G-6-P.

The formation of glucuronides from glucose and glycogen

In Fig. 5 (curve c) it was shown that UPPGA can be formed from glucose in the presence of the requisite enzymes and substrates. The dependence of the overall reaction from glucose to *o*-APG on the ATP concentration is shown in Fig. 6.

It will be seen that the rate of formation of *o*-APG is dependent upon the ATP concentration; in this case the ATP requirement is for G-6-P formation. The low rate of *o*-APG formation is again probably a reflection of the position of the phosphoglucomutase equilibrium.

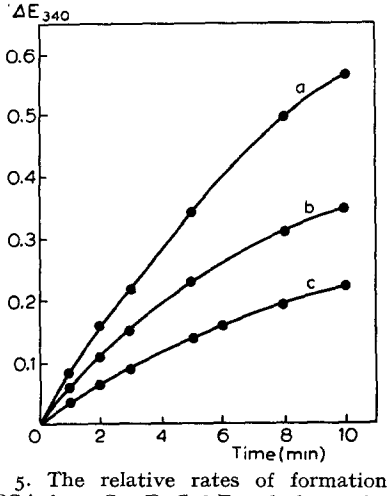

Fig. 5. The relative rates of formation of UPPGA from G-1-P, G-6-P and glucose in the presence of UTP. Curve (a) 0.5 μmole G-1-P, 0.2 μmole UTP, 0.5 μmole DPN, 0.5 mg Zwischenferment, 5 μmoles $MgCl_2$, 30 μl UPPG dehydrogenase and 0.1*M*-TRIS buffer pH 8.5 to 1.0 ml. Curve (b) 0.5 μmole G-6-P, 0.2 μmole UTP, 0.5 μmole DPN, 100 μl phosphoglucomutase, 5 μmoles $MgCl_2$, 10 μmoles cysteine, 0.5 mg Zwischenferment, 30 μl UPPG dehydrogenase and 0.1*M*-TRIS buffer pH 8.5 to 1.0 ml. Curve (c) 0.5 μmole glucose, 0.2 μmole UTP, 1.0 μmole ATP, 0.5 μmole DPN, 1 mg hexokinase, 100 μl phosphoglucomutase, 5 μmoles $MgCl_2$, 10 μmoles cysteine, 0.5 mg Zwischenferment, 30 μl UPPG dehydrogenase and 0.1 *M*-TRIS buffer pH 8.5 to 1.0 ml. Corrections made for controls run without UTP.

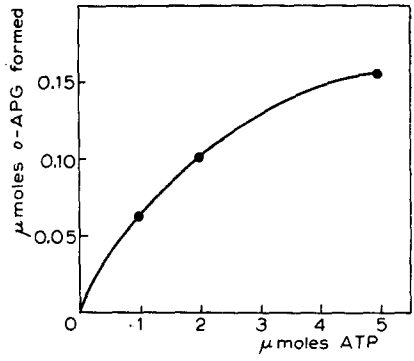

Fig. 6. The effect of ATP concentration on the overall formation of *o*-APG from glucose. Incubation mixtures contained 0.5 μmole glucose 2 mg hexokinase, 200 μl phosphoglucomutase, 5 μmoles $MgCl_2$, 10 μmoles cysteine, 0.5 μmole UTP, 1.0 μmole DPN, 25 μl glutamic dehydrogenase, 2 μ-moles α-oxoglutarate, 2.0 μmoles NH_4Cl, 0.5 mg Zwischenferment, 100 μl UPPG dehydrogenase, 0.1*M*-TRIS buffer pH 8.5 to 1.0 ml. and ATP as indicated. Reactions run for 40 min, heated for 30 seconds in a boiling water bath, cooled, centrifuged, the pH of the supernatant adjusted to pH 7.7 and UPPGA measured by *o*-APG formation.

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It was found possible in these experiments to substitute glycogen, phosphorylase and inorganic phosphate for the G-1-P thereby establishing a pathway from glycogen to glucuronide. A typical experiment is recorded in Fig. 7. It will be noted from this figure that the rate of formation of UPPGA from glycogen is almost as fast as from G-1-P and much faster than from G-6-P or glucose.

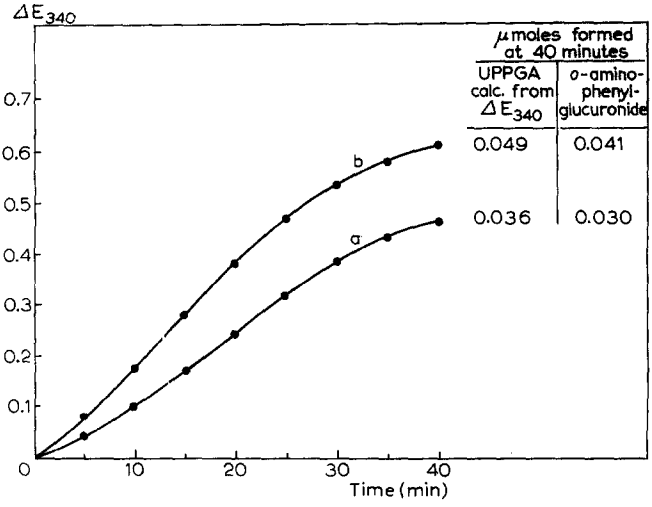

Fig. 7. The formation of UPPGA and *o*-APG from glycogen and the effect of UTP concentration thereon. Incubation mixtures contained 25 mg glycogen, 100 μl phosphorylase (equivalent to 4.5 g muscle), 10 μmoles cysteine, 0.1 μmole AMP (5'), 1 mg Zwischenferment, 5 μmoles $MgCl_2$, 1.0 μmole DPN, 30 μl UPPG dehydrogenase, 0.5*M*-phosphate buffer pH 7.8 to 1.0 ml and 0.1 μmole UTP (curve (a)) and 0.2 μmole UTP (curve (b)). After 40 minutes incubation the mixtures were treated as in Fig. 6 for the measurement of *o*-APG formation. Corrections were made for controls run without UTP.

DISCUSSION

The formation of glucuronides by liver slices was shown by LIPSCHITZ AND BUEDING[20] to be an aerobic process, inhibited by cyanide. These workers also found fluoride and iodoacetate to inhibit glucuronide formation at levels which did not greatly depress the oxygen uptake of the slices and concluded that phosphorylation reactions were important in glucuronide formation. The work of LIPSCHITZ AND BUEDING indicated that liver glycogen was a probably source of glucuronic acid and they suggested that 3-carbon intermediates were important in glucuronide formation, these compounds being more effective than glucose in stimulating glucuronide formation in liver slices from fasted animals. This observation however may only be an indication of the intermediate role of the hexose phosphates, the latter being formed more readily from 3-carbon compounds than from glucose due to the low hexokinase levels of liver[21,22]. Liver glycogen was implicated as a source of glucuronic acid by DZIEWIATKOWSKI AND LEWIS[23] who demonstrated a fall in liver glycogen simultaneous with glucuronide formation in the whole animal. A number of workers[24–30], using ^{14}C labelled glucose in both mammalian and bacterial systems, have demonstrated the conversion of glucose to glucuronic acid without cleavage of the carbon chain, while others[31,32] have indicated that preformed glucuronic acid is probably not a precursor of

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glucuronides. The implicating of the uridine pyrophosphoglycosyl compounds in glucuronide formation[1,5,33] has led to an elucidation of the probable pathway of glucuronide formation in liver, and the results of the present work on isolated enzyme systems indicates that the pathway may be represented as in Fig. 8.

In this system the important requirements are a supply of ATP and a DPN regenerating system in addition to the substrate glycogen. It was shown by SMITH AND MILLS[1] that cyanide, fluoride and iodoacetate have negligible effects on the transfer of glucuronic acid from UPPGA to the acceptor. In liver slices it is probable that the effects of these three inhibitors are on the ATP regenerating systems leading to a depletion of available ATP. In the present work it has been shown that the overall formation of glucuronides from glucose may be dependent upon the ATP supply; in this respect ATP will be required for the resynthesis of UTP from UPP as well as for hexose phosphate formation.

The central role of the uridine pyrophospho compounds of glucose and glucuronic acid allows the formation of an "active" form of glucuronic acid without free glucuronic acid existing as an intermediate. In the dehydrogenation step from UPPG to UPPGA

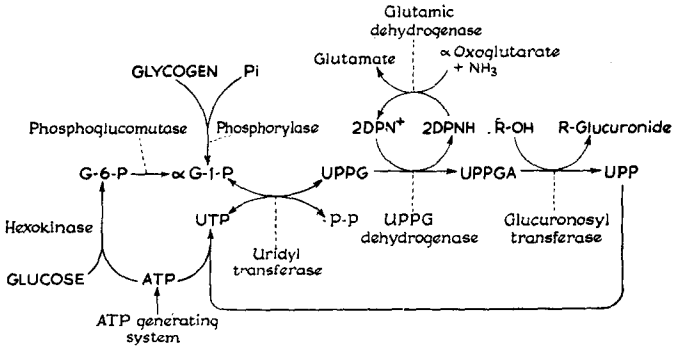

Fig. 8. The pathway of glucuronide formation.

the level of oxidised DPN will be a rate determining factor and here again the coupling of this step with the oxidative metabolism of the cell will allow maintenance of the correct level of DPN^+. It is probable that many other DPNH oxidising systems could substitute for the glutamic dehydrogenase system used in the present work. It would thus appear probable that liver glycogen is the ultimate source of glucuronic acid and that the direct formation from glucose is a minor route due to low liver hexokinase levels and the position of the phosphoglucomutase equilibrium.

It has been claimed by FISHMAN[34] that the enzyme β-glucuronidase is involved in glucuronide synthesis. The main arguments against this view are the lability of the glucuronosyl transferase as compared with β-glucuronidase and the fact that saccharo 1:4 lactone does not inhibit glucuronide formation at levels which completely inhibit β-glucuronidase[35]. FISHMAN AND GREEN[36] have recently demonstrated that β-glucuronidase may have a glucuronosyl transferring action and they have suggested[37] that this mechanism is important in glucuronide formation. While β-glucuronidase may, in fact, be active in transferring glucuronic acid from a glucuronide to another aglycone, primary formation of the glucuronide is obviously necessary and the most likely mechanism, based on all the available evidence, is that proposed in Figure 8.

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As will be seen from Figs. 1 and 2 the highest level of formation of *o*-APG is 85 % when based on DPN reduction during the formation of UPPGA from UPPG. It has previously been shown[1] that, when using chromatographically pure UPPGA, the level of *o*-APG formation approaches 100 %. In the present work the lower rate of *o*-APG formation may be due to breakdown of UPPGA by the enzyme preparations used. Some preliminary results indicate that when the UPPG dehydrogenase is allowed to act for shorter times the *o*-APG formed in such a conjugation reaction is closer to 100 %; similar findings have been reported by STROMINGER *et al.*[9].

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SUMMARY

A study has been made of the formation of uridine pyrophosphoglucuronic acid (UPPGA) and *o*-aminophenyl glucuronide (*o*-APG) in isolated enzyme systems. It has been shown that the conversion of uridine pyrophosphoglucose (UPPG) to UPPGA can be made independent of DPN concentration by using a DPNH oxidising system; by using such a coupled system, the conversion of UPPG to *o*-APG is about 85% of the UPPG present.

Coupling the UPPG dehydrogenase system with the uridyl transferase of MUNCH-PETERSEN, it was shown that the rate of UPPGA formation was dependent upon the UTP and G-1-P concentrations. Glycogen, phosphorylase and inorganic phosphate can be substituted for G-1-P with little reduction in the rate of UPPGA formation. The use of G-6-P and phosphoglucomutase, or glucose, ATP, hexokinase and phosphoglucomutase as substitutes for G-1-P gave a slower rate of UPPGA formation.

From these experiments a mechanism of glucuronide formation from glucose and glycogen is proposed and discussed in relation to other work on the mechanism of glucuronide formation.

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INFLUENCE DES CHLORURES ALCALINS SUR L'HYDROLYSE TRYPSIQUE DE LA LACTOGLOBULINE NATIVE ET DÉNATURÉE PAR LA CHALEUR

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Divers travaux ont permis de déterminer la nature des forces impliquées dans l'association d'un enzyme avec son substrat[1,2,3]. Les substrats utilisés avaient une structure chimique relativement simple. Plus récemment, STEINER[4], étudiant au moyen de la lumière diffusée l'association de la trypsine avec une autre protéine, l'inhibiteur du soja, en fonction du pH et de la force ionique, arrive à la conclusion que la formation du complexe implique la participation d'un groupe carboxyle. Une étude analogue a été appliquée par cet auteur au problème de la dimérisation de l'α-chymotrypsine[5].

Lorsque les forces d'attraction coulombiennes interviennent d'une manière appréciable dans l'association enzyme-substrat, on peut s'attendre à ce qu'une modification de la force ionique du milieu entraîne des variations dans l'affinité de l'enzyme pour son substrat; au contraire si les liaisons ioniques sont négligeables ou s'il y a compensation entre les attractions et les répulsions électrostatiques, on ne notera aucune variation sensible de l'affinité. Dans le présent travail, nous avons donc étudié quantitativement l'effet de l'augmentation de la concentration saline (principalement KCl et NaCl) sur l'hydrolyse des formes natives et dénaturées (*s* et *f* de BRIGGS ET HULL[6]) de la β-lactoglobuline. L'étude cinétique et thermodynamique permet de localiser l'effet des sels qui peut s'exercer soit au stade de la formation réversible du complexe intermédiaire, soit sur le processus de décomposition irréversible de ce complexe.

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