SUMMARY

1. A two- to threefold increase in guinea pig liver creatine occurs within 24 hr. after the withdrawal of food.

2. In rats the increase in liver creatine is less marked, but a pronounced creatinuria occurs coincident with a fall in blood sugar to 60–70 mg./100 ml.

3. Perfusion experiments have demonstrated that rat skeletal muscle creatine is reversibly sensitive to blood sugar levels.

4. The relation between creatine distribution, carbohydrate metabolism and electrolyte balance is discussed.

We are indebted to Professor J. H. Burn for advice and for extending to us the facilities of his Department, to Drs Coxon, Fisher and Parsons for much helpful discussion and to Mrs Lawrence for providing us with hypophysectomized rats. We should also like to thank Miss M. Cole and Mrs J. Kingerlee for skilled technical assistance.

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Uridine Compounds in Glucuronic Acid Metabolism

2. THE ISOLATION AND STRUCTURE OF 'URIDINE-DIPHOSPHATE-GLUCURONIC ACID'

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We have previously reported that liver contains a nucleotide composed of uridylic acid, labile phosphate and glucuronic acid in equimolecular proportions, and that the incubation of this substance with *o*-aminophenol or menthol in the presence of broken-cell liver suspensions results in the formation of the corresponding glucuronides (glucosiduronic acids) (Dutton & Storey, 1951, 1953). The enzyme system in the liver suspensions and some properties of the nucleotide have been described (Dutton & Storey, 1954).

The present paper deals with the isolation of the pure nucleotide and a study of its degradation products under various conditions. The results confirm an earlier suggestion (Dutton & Storey, 1953) that its structure resembles the uridine diphosphate compounds isolated by Caputto, Leloir, Cardini & Paladini (1950), Cabib, Leloir & Cardini (1953) and Park (1952*a*, *b*), and that it may be regarded as 'uridine-diphosphate-glucuronic acid'.

Since the new nucleotide appears to be a substrate in the enzymic process, throughout both this and the preceding paper (Dutton & Storey, 1954) we have used the term 'factor' rather than 'cofactor' or 'coenzyme', which imply a function which is, as yet, unproved.

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EXPERIMENTAL

Methods

Measurement of activity of factor and of glucuronide synthesis. The course of the purification of the factor was followed by measuring its activity in the liver-suspension system in presence of *o*-aminophenol or (−)-menthol as previously described (Dutton & Storey, 1954). Recently, it was found advantageous to reduce all volumes to approx. one-fifth, to permit the use of smaller amounts of the factor. Aminotrishydroxymethylmethane (THAM) buffer was used in preference to phosphate, since it does not form a precipitate with Mg^{2+}, and glucuronide synthesis appeared to be equally good in both media. Each reaction vessel received 0·04 ml. 0·5M THAM buffer (pH 7·4) containing 0·15M-$MgCl_2$, the final incubation volume being 0·6 ml. The 1 cm. microcells of the Spekker absorptiometer were used in reading the extinctions.

Phosphorus determinations. Inorganic P was determined by the method of Fiske & Subbarow (1925). For labile P, the sample was made N with HCl and heated at 100° for the required period; inorganic P was then determined. Total P was estimated by the method of King (1946). These methods could be used satisfactorily for measuring 0·1–2·0 μg. P by suitably reducing the volumes of all solutions.

Uridylic acid. This was measured from its extinction at 260 mμ. in 1 cm. cells with a Unicam SP. 500 spectrophotometer, using the data of Smith & Markham (1950).

Glucuronic acid. Glucuronic acid in the pure factor was determined by the naphthoresorcinol reaction as modified by Paul (1951; see also Dutton & Storey, 1954), but the ethyl acetate extraction was omitted. D-Glucurone (Roche Products) was used as the standard.

Reducing power. This was measured by the method of Folin & Malmros (1929).

Paper chromatography. This was carried out in all-glass tanks by the ascending method whenever possible, but if greater resolution was required the descending method was used. Except where specifically stated, chromatograms were run at room temperature. Whatman no. 541 paper was chosen for its low ash content, and it was satisfactory with all solvent systems without any preliminary washing. Ultraviolet photography of developed chromatograms was carried out as described by Markham & Smith (1949, 1951). Phosphate compounds were detected with the molybdate reagent of Hanes & Isherwood (1949). Identifications of spots were made by reference to standard compounds rather than by measurement of R_F values.

Materials

Uridine 5′-phosphate and uridine 5′-pyrophosphate were synthetic products kindly given by Professor Sir Alexander Todd, F.R.S., and Dr G. W. Kenner. Yeast uridylic acid (L. Light and Co.) and cytidylic acid (a gift from Dr C. M. Mauritzen) were presumably mixtures of the 3′ and 2′ isomers, but for convenience they will be referred to as the 3′-phosphates. Adenosine 5′-phosphate (L. Light and Co.) was pure as judged by paper chromatography and determinations of total P and absorption at 260 mμ.

α- and β-D-glucuronic acid 1-phosphates, as previously described (Dutton & Storey, 1954), were gifts from Dr C. A. Marsh and Dr O. Touster, respectively. α-D-Glucose 1-phosphate was presented by Dr A. B. Roy.

Dried cobra (*Naia naia*) venom and antiserum were the materials described by Johnson, Kaye, Hems & Krebs (1953), and were generously presented by Dr M. A. G. Kaye.

Russell's viper venom. 'Stypven' (Burroughs Wellcome and Co.) was used.

RESULTS

Isolation of the pure factor

Extraction from liver. The procedure already described (Dutton & Storey, 1954) for obtaining a crude preparation of the factor was modified by extracting the disrupted rabbit liver with boiling water (50–80 ml./100 g. liver) rather than with a solution of trichloroacetic acid, to avoid any possible destruction of the activity by prolonged contact with strong acid. The ice-cold, strained extract was brought to pH 2 with 20% (w/v) trichloroacetic acid. An equal volume of ethanol was then added and the precipitate of glycogen and protein was centrifuged down. This and subsequent operations were carried out with all solutions chilled to 0° whenever possible.

Fractionation of barium salts with ethanol. The solution was immediately brought to pH 8·2–8·5 (glass electrode) with NaOH and fractionated according to procedure *B* of LePage & Umbreit (1945). The barium salts were precipitated by adding 3 ml. M barium acetate/100 g. wet weight of liver, and ethanol to a total concentration of 80% (v/v). After chilling for 30 min. the mixture was centrifuged and the supernatant discarded. The precipitate was dissolved in dilute HCl and the solution readjusted to pH 8·2–8·5. The precipitate of water-insoluble barium salts thus obtained was reprecipitated by dissolving in HCl and readjusting the pH, and 4 vol. of ethanol was added to the combined supernatants from these two precipitations. The active factor was present in the resulting precipitate of water-soluble ethanol-insoluble barium salts. The other barium-salt fractions did not show any activity, either singly or in combination. Barium was subsequently removed as $BaSO_4$, the solution was adjusted to pH 7·4 with NaOH and then concentrated *in vacuo* at 50° to a convenient volume (about 150 ml. for a batch of 7–15 rabbits).

Phenol extraction. The solution had a bright greenish-yellow tint at this point, and unless most of this colouring matter was removed traces were carried through the subsequent purification and were present in the final product. The solution was therefore saturated with phenol and extracted thrice with one-sixth vol. phenol saturated with water, followed by four washings with 0·25 vol. peroxide-free diethyl ether. The ether was then removed *in vacuo* at 50°.

Chromatography on Dowex-1. This step was based on the method of Cohn & Carter (1950) for adenosine phosphates, but to obtain the maximum separation of the factor from ultraviolet-absorbing impurities a different range of eluting solutions were used.

A column (1 cm. × 1 cm.^{2}) of Dowex-1 anion-exchange resin was washed with 2N-HCl until all ultraviolet-absorbing impurities had been removed and then with water until the effluent was neutral. The solution of the factor (150 ml.) was first brought to pH 8·5 with concentrated NH_3 solution (sp.gr. 0·88) and any precipitate removed before it was added to the column at room temperature. The flow rate was kept at 2 ml./min. by pressure from a mercury manometer. Distilled water (40 ml.) was passed through the column, followed by 0·02M-NaCl in 0·01N-HCl until the issuing solution was acid to litmus. No activity was eluted

under these conditions. The following fractions were then collected manually (flow rate 3 ml./min.): 4 × 25 ml. with 0·02 M-NaCl in 0·01 N-HCl as eluent; 8 × 10 ml. with 0·05 M-NaCl in 0·01 N-HCl; 6 × 10 ml. with 0·1 M-NaCl in 0·01 N-HCl and 4 × 10 ml. with 0·2 M-NaCl in 0·01 N-HCl. All these fractions were cooled in ice-water, neutralized immediately, and 0·4 ml. samples tested in the liver-suspension system for the presence of the factor. Since paper chromatography of solutions from the previous stage had suggested a relationship between activity and absorption at 260 mμ., the fractions were also examined for absorption at that wavelength. The relationship between activity and absorption is shown in Fig. 1. The activity in zone *A* suggests the constant elution of a small amount of the factor during the rapid washing out of inactive material. In zone *B*, during the rapid rise to the pronounced peak and the subsequent fall, the activity and absorption maintain a fairly constant ratio.

According to Smith & Markham (1950), purines are liberated from their ribonucleotides by hydrolysis with N-HCl for 1 hr. at 100°, whereas pyrimidine ribonucleotides are virtually unaffected. Hydrolysis of samples of the various pooled fractions under these conditions and subsequent chromatography in *iso*propanol—HCl (Wyatt, 1951), adjusted to give maximum separation of purines and pyrimidines, revealed that absorption in zone *P* was due to adenine compounds (deduced from the identical R_F value of the released purine with authentic adenine, and from its absorption curve after elution (Ploeser & Loring, 1949)), that in zone *A* to a mixture of adenine and pyrimidine nucleotides and in zone *B* almost entirely to pyrimidine nucleotides.

The combined *B* fraction (60 ml.) was concentrated *in vacuo* at 50° to 0·33 vol. and fractionated through the barium

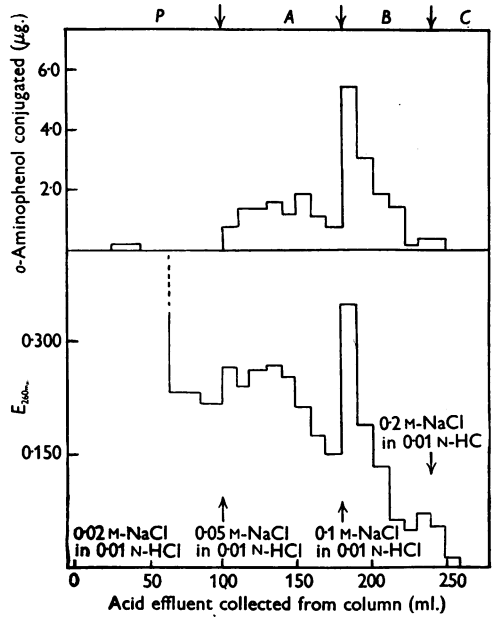

Fig. 1. Ion-exchange chromatography of water-soluble ethanol-insoluble barium salts from rabbit liver, showing the relationship between ultraviolet absorption (lower diagram) and glucuronide formation (upper diagram) in acid effluent from Dowex-1 column.

salts as described previously to avoid salt effects in the subsequent paper chromatography. The precipitate of water-soluble ethanol-insoluble barium salts was then freed of Ba^{2+} and ethanol and concentrated *in vacuo* at 30° to 2–3 ml. of colourless solution.

Paper chromatography. When a band of this solution was chromatographed by the ascending method in methanol–water–conc. (0·88) NH_3 (60:30:10, by vol.) (Bandurski & Axelrod, 1951) for 18 hr. at 2°, only one ultraviolet-absorbing zone was apparent, and this coincided with the activity. Chromatography in the acid solvent of the same authors, methanol–formic acid (88%, w/v)–water (80:15:5, by vol.), gave rise to two bands with strong ultraviolet absorption, and only the more slowly moving one contained the factor. (A third, very weak band of absorption was sometimes detectable between the two other bands.) However, the use of a strongly acid solvent resulted in a considerable loss of activity and poorly defined bands, presumably owing to decomposition of the factor during chromatography. These difficulties were avoided with a weakly acidic solvent system. After development for 18 hr. at 2° in ethanol–M ammonium acetate, pH 5·5 (60:40, by vol.), the slower-moving band was cut out and eluted with water by capillarity. When all the material had been put through this process the factor was freed of ammonium acetate by precipitation as the Ba salt. It was then converted into the potassium salt and stored at −20°, at which temperature it was stable for long periods.

Various preparations of the slower-moving band behaved as a single substance in the following solvents at 2° (all proportions are by vol.): *n*-propanol–concentrated NH_3–water (60:10:30) (Hanes & Isherwood, 1949); methanol–conc. NH_3–water (60:10:30); *iso*propanol–$(NH_4)_2SO_4$ (1% w/v) (60:40) (Anand, Clark, Hall & Todd, 1952); ethanol–M ammonium acetate, pH 4·0 (60:40); methanol–formic acid (88%, w/v)–water (80:5:15). Consistent analytical results (see later) were obtained with several different preparations of this band, and there does not appear to be any evidence for the presence of appreciable amounts of contaminating materials.

Comments on the method. Since it was essential to remove the livers immediately after the death of the rabbits (Dutton & Storey, 1954), it was usual to prepare boiled extracts from about six livers at a time, whereas the remainder of the purification was more conveniently carried through with several such batches. Later, the ion-exchange procedure was modified for handling increased amounts of material by the use of a larger column (3 cm. × 1·8 cm.²) and different volumes of eluting solutions. For the material from fifteen rabbits (150 ml.), once the effluent was acid 150 ml. of 0·02 M-NaCl in 0·01 N-HCl and 150 ml. of 0·05 M-NaCl in 0·01 N-HCl preceded the 3 × 20 ml. of 0·1 M-NaCl in 0·01 N-HCl required to elute the factor, the flow rates remaining as before. To minimize losses on the column the process was carried out as rapidly as possible, and the factor was in contact with the resin for only 3 or 4 hr. It seems probable that failure to observe this condition was the cause of the difficulties recently reported with this procedure (Smith & Mills, 1954). After the column of resin had been regenerated with 2 N-HCl, it could be used again.

Yield. In a preparation from thirty rabbits (approx. 2·5 kg. liver), 65% of the activity of the boiled aqueous extract was recovered after the barium–ethanol fractionation and 20% after the resin treatment. The final yield was 15% and amounted to about 8 mg. of the pure factor.

Whereas the weight of the pure factor was determined by measurement of the absorption at 260 mμ., the percentages are only approximate, since they were derived by enzymic assay. In general, the weight of the factor isolated represented 10–20% of the boiled-extract activity. The extraction of the finely minced tissue with boiling water was itself very inefficient, but the volume of water had to be severely restricted to keep operations within the limits of the facilities available. Indeed, much larger amounts of the factor are present in liver than the above figures suggest. Direct assays of boiled extracts of rabbit and mouse liver, using a high water–tissue ratio of 10:1, gave values of the order of 20 mg. (34 μmoles) factor/100 g. liver, when a correction is made for the transfer of the glucuronic acid from the factor to the *o*-aminophenol being only 50% complete (see later). This figure is in good agreement with that quoted by Smith & Mills (1954) for guinea pig liver, if a similar correction were applicable to their conditions of assay.

Where the pure factor was not required, the acetone-precipitated 'crude factor' (Dutton & Storey, 1954), or a similar preparation made from a boiled extract, has been found useful for many purposes. A subsequent barium–ethanol treatment gave further purification without great loss of active material.

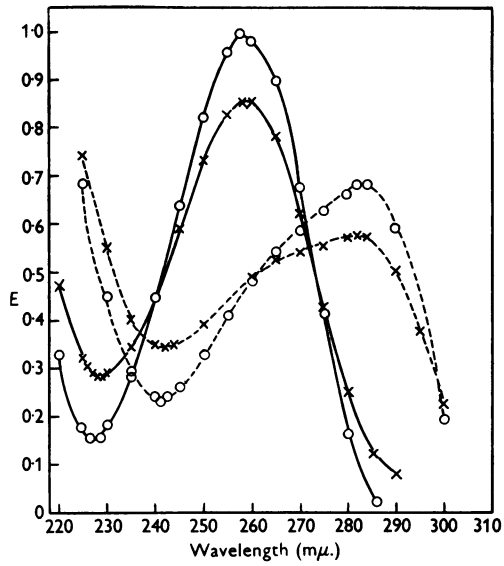

Fig. 2. Absorption spectrum of uracil (circles) and of pyrimidine from factor (crosses) in 0·1 N-HCl (full lines) and in 0·1 N-KOH (broken lines).

Investigations on the structure of the factor

Presence of uracil. As has already been indicated, on paper chromatograms the region which showed activity always coincided with that having ultraviolet absorption. The material eluted from this area exhibited an absorption spectrum resembling that of a uridine-containing nucleotide (Ploeser & Loring, 1949; Caputto *et al.* 1950) with a maximum at 261 mμ. and minimum at 229–230 mμ. in 0·1 N-HCl.

To prepare the free pyrimidine, the solution was transferred to a heavy-walled glass tube and blown to dryness at 70°. Formic acid (0·35 ml. of 98%, w/v) was added and the tube was sealed. After heating at 175° (5 hr. were found necessary to free the base completely, cf. Chargaff *et al.* 1950), the solution was chromatographed by the method of Wyatt (1951) with adenine, guanine, cytosine and uracil as markers. The only ultraviolet-absorbing area was at the uracil level, and after elution it gave the characteristic uracil absorption curves (Hotchkiss, 1948; Ploeser & Loring, 1949) with maxima and minima in acid and alkaline solutions virtually identical with those of authentic uracil (Fig. 2). Since uracil was the only ultraviolet-absorbing component in the factor, and since it was present as uridylic acid (see later), the measurement of the absorption at 260 mμ. was used in calculating the concentration of solutions of the factor, using the value $\epsilon_{260\ m\mu.} = 9890$ for uridylic acid (Smith & Markham, 1950); all molar ratios for the other constituents (Table 1) were based on the assumption that the factor contained one molecule of uridylic acid.

Phosphate. After the pure factor had been run as a band for 18 hr. at 2° in methanol–conc. NH_3–water (60:10:30, by vol.), longitudinal strips were sprayed for phosphate and the remainder of the chromatogram was cut latitudinally into narrow equal strips, which were then added to the liver-suspension system in a complementary series of flasks. The position of the strips giving rise to glucuronide formation corresponded exactly with the phosphate-containing zone and with the ultraviolet absorption.

The factor was found to contain two molecules of phosphorus per molecule of uridylic acid (Table 1), and when it was heated in N-HCl at 100° half the phosphorus was liberated in the inorganic form ('labile P') in 10 min., whilst the remainder was released much more slowly (Fig. 3).

Glucuronic acid. A definite, positive naphthoresorcinol reaction was not obtained with crude preparations before ion-exchange chromatography, but afterwards it was

Table 1. *Molar ratios of various components of the factor referred to uridylic acid*

	Molar ratio (uridylic acid = 1·00)				
Preparation no.	1	2	3	4	5
Total P	2·12	2·00	—	—	2·15
Labile P	1·10	0·90	—	—	1·07
Glucuronic acid (naphthoresorcinol reaction)	0·88	0·91	—	—	—
Reducing substance (calc. as glucuronic acid)	—	—	0·92	0·95	—

readily evident. With paper chromatography, the area giving the reaction always coincided with the zone of ultraviolet absorption, phosphate and activity. The naphthoresorcinol-positive substance behaved identically with authentic glucuronic acid in paper chromatography and ionophoresis experiments. Different preparations showed a constant proportion of approximately one molecule of glucuronic acid to one of uridylic acid (Table 1). An identical ratio was found with the orcinol method of Meijbaum (LePage & Umbreit, 1945), using D-glucurone as the standard. Uridine 5′-pyrophosphate did not give any reaction for pentose under these conditions.

Reducing substance. It has been previously shown (Dutton & Storey, 1954) that the activity of the factor is completely lost after heating at 100° for 8 min. in 0·1 N-HCl. Now Caputto *et al.* (1950) found that treatment of uridine-diphosphate-glucose with 0·01 N acid at 100° for 5 min. split off the glucose quantitatively. When the pure factor was heated for 10 min. in 0·1 N-HCl at 100°, a reducing substance was likewise liberated in an amount equivalent to one molecular proportion, when calculated as glucuronic acid (Table 1). The reducing value was not increased by further heating.

Products of hydrolysis by N *acid*

As has been shown above, when the factor was heated with N-HCl at 100° for 15 min. one molecule of inorganic phosphate was liberated. The other breakdown products were examined by paper chromatography.

After hydrolysis of the factor in N-H_2SO_4 at 100° for 15 min., the solution was cooled and brought to pH 3–4 with $Ba(OH)_2$. The $BaSO_4$ was centrifuged down and washed, and the combined supernatants were neutralized with 0·1 N-KOH before concentrating to a small volume *in vacuo* at 25–30°. The solution was then chromatographed with untreated factor, uridine 5′-pyrophosphate, uridine 5′-phosphate and uridine 3′-phosphate for comparison (Fig. 4*a*, *b*). The ethanol–boric acid system slowed down the uridine 5′-phosphate very markedly compared with the 3′ isomer, presumably because of complex formation (cf. Cohen & Scott, 1950). In the two solvent systems shown, and also in *iso*propanol–$(NH_4)_2SO_4$ (1%, w/v) (60:40, by vol.), the only ultraviolet-absorbing substance present in the hydrolysate travelled exactly as did uridine 5′-phosphate.

When chromatograms of the hydrolysate in ethanol–M ammonium acetate, pH 5·5 (60:40, by vol.) were treated with the aniline oxalate reagent (Partridge, 1949), an orange spot was found at the level of glucurone and a faint smear at that of glucuronic acid. Formation of the lactone would be expected during the N acid hydrolysis if glucuronic acid were present. Galacturonic acid did not form a lactone under such conditions (cf. Partridge, 1948), and gave only a brown spot at the glucuronic acid level.

It thus appeared that treatment of the factor with N-H_2SO_4 for 15 min. at 100° gave rise to uridine 5′-phosphate, inorganic phosphate and glucuronic acid only. No other compounds containing phosphorus, showing ultraviolet absorption or giving a colour with aniline oxalate were detected.

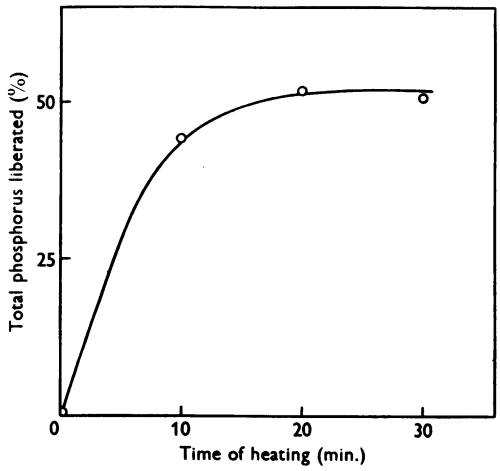

Fig. 3. Liberation of inorganic phosphate from the factor on heating in N-HCl at 100° for varying periods of time.

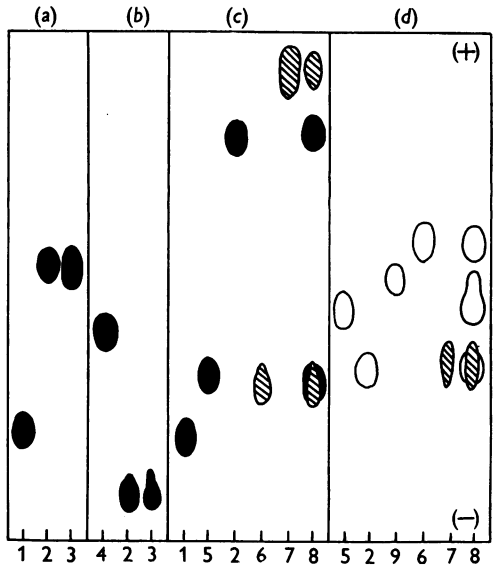

Fig. 4. Paper chromatography and ionophoresis of hydrolysis products of the factor. All origins at base line. Chromatogram solvents allowed to drip off paper. Ultraviolet-absorbing areas are black, areas giving colour with aniline oxalate are cross-hatched and those showing phosphate are unshaded. (*a*) Chromatography in ethanol–M ammonium acetate, pH 4·0 (60:40, by vol.) for 17 hr. (*b*) Chromatography in ethanol–boric acid (0·6%, w/v) (80:20, by vol.) for 17 hr. (*c*) Chromatography in ethanol–M ammonium acetate, pH 4·5 (65:35, by vol.) for 20 hr. (*d*) Ionophoresis in 0·05 M sodium acetate, pH 5·9, at potential of 3·5 v/cm. for 18 hr. 1, pure factor; 2, uridine 5′-phosphate; 3, product of hydrolysis of factor with N-H_2SO_4 at 100° for 15 min.; 4, uridine 3′-phosphate; 5, uridine 5′-pyrophosphate; 6, β-D-glucuronic acid 1-phosphate; 7, glucuronic acid (potassium salt, Corn Products Ltd.); 8, products of hydrolysis of factor with 0·1 N-H_2SO_4 at 100° for 7 min.; 9, orthophosphate.

Further identification of uridine 5′-phosphate

The uridylic acid was prepared by hydrolysing the factor in N-HCl for 15 min. at 100° and treating with barium acetate at pH 8·2. After removal of the inorganic phosphate, the nucleotide was precipitated with ethanol and converted into the K salt by treatment with K_2SO_4. The solution was then concentrated *in vacuo*.

Hydrolysis by Russell's viper venom. Gulland & Jackson (1938) first showed that the venom of Russell's viper and certain other snakes was a potent source of an enzyme that hydrolysed 5′-nucleotides, but that it was without effect on 3′-nucleotides. Such venoms have been subsequently used (Park, 1952*b*; Cabib *et al.* 1953; Cabib & Leloir, 1954) to prove that the uridylic and guanylic acids isolated from the corresponding diphospho-glycosyl compounds are 5′-phosphates. In our experiments adenosine 5′-phosphate was completely hydrolysed by Russell's viper venom, whilst uridine 5′-phosphate and the uridylic acid from the factor were hydrolysed to the extent of approximately 60–95%. Cytidine 3′-phosphate and uridine 3′-phosphate were not hydrolysed even after 2 hr. incubation. Two results are shown in Table 2. They show clearly that the uridylic acid from the factor resembles the nucleotides with the phosphate esterified at the 5′ position.

Copper-complex formation. The micromethod described by Caputto *et al.* (1950) and by Park (1952*b*) was adapted for amounts of nucleotide of the order of 2% of that used by these authors. The sample (0·5 ml. containing 45–80 μm-moles nucleotide) was allowed to react, with occasional stirring, for 2 hr. in a 3 ml. microcentrifuge tube with 0·1 ml. 10% (w/v) $Na_3PO_4, 12H_2O$ and 0·3 mg. copper phosphate. After centrifuging, 0·3 ml. of the supernatant was mixed with 0·1 ml. 0·1% (w/v) diethyl dithiocarbamate and the colour extracted by shaking with 0·7 ml. amyl alcohol. Any turbidity in the upper layer was removed by adding 0·05 ml. ethanol and mixing carefully with a capillary pipette. The colours were read in the Spekker absorptiometer using Ilford filter no. 601 (transmitting 385–470 mμ.).

The results showed some variation probably owing to the small scale of working, but nevertheless the uridylic acid from the factor closely resembled adenosine and uridine 5′-phosphates in its copper-binding capacity (Table 3). The 3′-phosphates were unable to solubilize appreciable amounts of copper.

Table 2. *Action of Russell's viper venom upon uridylic acid from the factor and upon other nucleotides*

Each tube contained 12 μg. venom, 0·05 ml. 0·5 M THAM buffer, pH 7·40 and substrate (final concentration in each case about 0·1 mM) in a total volume of 0·35 ml. Incubation was for 1 hr. at 37°. The reaction was stopped with 0·1 ml. trichloroacetic acid (20%, w/v) and 0·05 ml. of each of the Fiske & Subbarow (1925) reagents were then added. In the controls venom was added after incubation. Phosphate standards were carried through the complete procedure.

Substrate	P liberated (%) Expt. 1	Expt. 2
Adenosine 5′-phosphate	100	—
Uridine 5′-phosphate	73	71
Uridylic acid (from factor)	82	58
Uridine 3′-phosphate	0	—
Cytidine 3′-phosphate	0	0

Table 3. *Copper-binding capacity of various nucleotides*

Nucleotide	μm-moles Cu bound/μm-mole nucleotide
Adenosine 5′-phosphate	0·59
Uridine 5′-phosphate	0·35
Uridylic acid (from factor)	0·38
Uridine 3′-phosphate	0·05
Cytidine 3′-phosphate	0·00

Products of hydrolysis by 0·1 N *acid*

A solution of the factor was heated at 100° in 0·1 N-H_2SO_4 for the 7 min. necessary for complete inactivation (cf. Dutton & Storey, 1951, 1954), and it was then neutralized and concentrated as previously. Chromatography in ethanol–M ammonium acetate, pH 4·5 (65:35, by vol.) showed two ultraviolet-absorbing areas corresponding with uridine 5′-phosphate and uridine 5′-pyrophosphate (Fig. 4*c*) in the ratio of approximately 1:0·8, and a trace of inorganic phosphate. In addition, the aniline oxalate reagent revealed two areas corresponding with glucuronic acid 1-phosphate and glucuronic acid in rate of travel and shade of colour (pink and brown, respectively). Both areas gave a positive naphthoresorcinol reaction for glucuronic acid, with a ratio of approximately 1:0·8. Unfortunately, the slower glucuronic acid 1-phosphate spot coincided exactly with uridine 5′-pyrophosphate in this and several other solvents, so that the area contained more phosphorus than corresponded with the ultraviolet absorption.

A more satisfactory separation of the hydrolysis products was obtained by paper ionophoresis in an apparatus of the Durrum (1950) type using Whatman no. 541 paper. After the paper had been sprayed with 0·05 M sodium acetate buffer, pH 5·9, a potential of approximately 3·5 v/cm. was applied for 18 hr. Examination of the strip for phosphate showed the following compounds, in order of increasing rate of travel: uridine 5′-phosphate, uridine 5′-pyrophosphate, inorganic phosphate (a trace) and glucuronic acid 1-phosphate (Fig. 4*d*). With aniline oxalate, the orange-brown spot of glucuronic acid was noted at the uridine 5′-phosphate level, and the characteristic rosy-pink of glucuronic acid 1-phosphate coincided with the fastest-moving phosphate-containing area. Both these regions gave a positive Tollens naphthoresorcinol reaction.

Although it was not feasible to characterize the glucuronic acid phosphate further, other considerations confirmed its formulation as the 1-ester. An acyl phosphate linkage at C-6 would also have been easily hydrolysable, but it would not have explained the increase in reducing power after acid hydrolysis (see later). The recent observation of Schmitz, Potter, Hurlbert & White (1954), that during the ion-exchange chromatography of the acid-soluble fraction of rat liver on Dowex-1 formate resin uridine 5′-pyrophosphate and a substance apparently identical with the factor were eluted together, also indicated that the carboxyl group must be free since otherwise they would not have had similar net charges. Positions 2–4 seem highly improbable, as such phosphate esters would presumably be relatively stable to hydrolysis in N acid. It has not yet been possible to show

whether the glucuronic acid 1-phosphate is the α or the β isomer, since they travelled at identical rates in ionophoresis experiments over a considerable pH range, and with paper chromatography any difference was too small to be demonstrated with certainty.

Since uridine 5′-pyrophosphate, uridine 5′-monophosphate and glucuronic acid 1-phosphate were identified as products of mild acid hydrolysis of the factor, then it follows that breakdown must have taken place at two different bonds, since there are only two molecules of P/molecule of uridylic acid. The only structure which will satisfy all the experimental findings is one in which uridine 5′-phosphate is joined to the glucuronic acid 1-phosphate through a pyrophosphate linkage. This type of structure could account satisfactorily for the slow destruction of the activity of the factor even with large amounts of a purified alkaline phosphatase preparation, whereas it was destroyed rapidly by crude tissue suspensions (Dutton & Storey, 1954).

Inactivation by snake venoms

Confirmatory evidence for the proposed structure was sought in a study of the action of certain snake venoms on the factor. Kornberg & Pricer (1950) have described a nucleotide pyrophosphatase in potatoes, but the preparation of the purified enzyme appeared difficult. A similar enzyme which split diphosphopyridine nucleotide (Chain, 1939; Zeller, 1951) and certain dinucleotide pyrophosphates (Christie, Elmore, Kenner, Todd & Weymouth, 1953) was demonstrated in various snake venoms. Cobra (*Naia naia*) venom contains a nucleotide pyrophosphatase splitting both diphosphopyridine nucleotide and coenzyme A (personal communication from Dr M. A. G. Kaye). We have therefore studied the action on the factor of Russell's viper and cobra venoms, neither of which liberated inorganic phosphate from α-glucose 1-phosphate or the α- and β-glucuronic acid 1-phosphates. The results of these experiments confirmed the structure proposed for the factor. Both Russell's viper and cobra venoms destroyed the activity of the factor on incubation at pH 7·4 in THAM buffer containing Mg^{2+} for 30 min. at 37°. Liberation of inorganic phosphate was much slower (Table 4), and was always less than 50% of the total phosphate, strongly suggesting that cleavage first took place at a pyrophosphate bond and that the liberated uridine 5′-phosphate was acted upon by the 5′-nucleotidase present. A similar, incomplete liberation of phosphate was observed when uridine 5′-phosphate itself was the substrate. The release of phosphate from both the factor and uridine 5′-phosphate was inhibited by antiserum, which was found by Johnson *et al.* (1953) to affect the 5′-nucleotidase more strongly than the pyrophosphatase. Support for the idea that a glucuronic acid 1-phosphate was liberated initially was provided in Expt. 3 of Table 4. After the 150 min. hydrolysis period 'labile P' was determined on the digest. The 'labile P' liberated (25·2 μm-moles) was in good agreement with the theoretical (27·6 μm-moles), showing that the P liberated by the enzymic hydrolysis (24·2 μm-moles) must have corresponded with the acid-stable P.

Inactivation by alkali

We have previously found (Dutton & Storey, 1954) that the factor is inactivated when heated with 0·1 N acid or alkali for 8 min. at 100°. In the present paper we have described the quantitative liberation of a reducing substance from the factor on heating at 100° with 0·1 N-HCl for 10 min. However, it was found that even without a preliminary acid hydrolysis the factor still showed 75% of the total reducing value; but since an approximately equal loss of activity was brought about merely by heating under the alkaline conditions (0·022 M-Na_2CO_3) used in the Folin & Malmros (1929) determination of reducing power, this result was not necessarily at variance with the proposed structure of the factor, where the glucuronic acid is linked in the 1-position. If the reducing value was determined after first heating with 0·1 N-KOH for 10 min. at 100° it was less than that of the untreated material and even subsequent acid hydrolysis (0·1 N-HCl for 8 min. at 100°) did not raise it to the maximum attainable value. This diminution in the rate of release of reducing substance by acid after alkaline treatment was also observed by Caputto *et al.* (1950) and Paladini & Leloir (1952) with uridine-diphosphate-glucose (UDPG). These authors presented evidence for the formation of uridine 5′-phosphate and a cyclic glucose monophosphate, probably esterified at positions 1 and 2, as the primary decomposition products. With the factor it has not yet been possible to show that a glucuronic acid phosphate ester is split off. After 4 hr. at 15° or 30 sec. at 100° in 9 N-NH_3 there was no chromatographic alteration detectable in the factor when run in the ethanol–M ammonium acetate, pH 7·5 (75:30, by vol.) solvent of Paladini & Leloir (1952) and subsequently examined for phosphate. In the ethanol–concentrated NH_3 (75:30, by vol.) solvent of the same authors the factor also gave only a single spot. Nevertheless, the factor and UDPG are both inactivated when chromatographed at room temperature in ammoniacal solvents. Further investigations of the action of alkali were postponed until larger amounts of the factor were available.

Table 4. *Liberation of inorganic phosphate from the factor by the action of snake venoms*

Each tube received 0·5 M THAM buffer, pH 7·4 (0·04 ml.) containing 0·015 M-$MgCl_2$, with other additions as shown, the total volume being 0·46 ml. Incubation was at 37°. The remainder of the procedure was as in Table 2.

	Russell's viper venom		Cobra venom					
	Expt. 1	Expt. 2	Expt. 3			Expt. 4		
Factor (μm-moles)	55·2	31·1	27·6			27·6		
Venom (μg.)	13	25	5			5		
Time of incubation (min.)	40	100	60	150	150	90	210	210
Antiserum (μg.)	—	—	—	—	20	—	—	20
P released (μm-moles)	23·0	20·4	21·6	24·2	15·5	11·6	16·5	0
Uridylic acid hydrolysed (%)	42	66	78	88	56	41	60	0

Nature of the reaction

In a previous paper (Dutton & Storey, 1954) we described some features of the reaction which took place in presence of the factor, an acceptor (*o*-aminophenol or (−)-menthol) and a liver suspension. In contrast with glucuronide formation in liver slices (Storey, 1950), the reaction was anaerobic and was unaffected by cyanide. It was also very rapid, being about 75% complete within 15 min. We have furthermore found that, provided the *o*-aminophenol is in large (approximately fivefold) excess, the amount of glucuronide formed is proportional to the concentration of the factor, indicating that the factor is a substrate rather than a cofactor. Since the factor contains glucuronic acid, it seems likely that the reaction involves merely the transfer of glucuronic acid from the factor to the acceptor (ROH) according to the equation:

$$\text{UDPglucuronic acid} + \text{ROH} \rightarrow \text{UDP} + \text{R–O—glucuronic acid},$$

where UDPglucuronic acid, UDP and R–O—glucuronic acid represent the factor, uridine 5′-pyrophosphate and the glucuronide, respectively.

Unfortunately, further investigation of the nature of the reaction was greatly hindered by the labile nature of the enzyme system and its association with insoluble cytoplasmic particles (Dutton & Storey, 1954). Only crude tissue preparations could therefore be used, either a whole-liver suspension or the supernatant prepared from this by centrifuging for 5 min. at 700 *g* ('centrifuged suspension'). The amount of glucuronide formed was only 40–60% (average 50%) of that required by the above equation, but as it could not be increased appreciably by varying the reaction conditions, it is unlikely that an equilibrium was being reached. This was further investigated by incubating UDP (0·05 mM) and *o*-aminophenylglucuronide (0·07 mM) with the centrifuged suspension in presence of saccharate (0·1 mM) as a β-glucuronidase inhibitor (cf. Dutton & Storey, 1954), but it was not possible to detect any disappearance of the glucuronide. The low yield of glucuronide might at least partially be explained by destruction of the factor by the liver suspension, since it lost 30% of its activity in 8 min. and 60% in 20 min. when incubated with the centrifuged suspension in the absence of *o*-aminophenol. The only degradation product which showed ultraviolet absorption and was detectable on paper chromatograms was uridine. UDP itself was apparently completely degraded to uridine with great rapidity even by preparations of the insoluble particles alone.

DISCUSSION

Although a full chemical characterization of the factor was impracticable with the amounts of material available, the present results appear to show without much doubt that it is a nucleotide composed of uridine 5′-phosphate and glucuronic acid 1-phosphate linked by a pyrophosphate bond. Our earlier proposal (Dutton & Storey, 1953) that it was structurally analogous to UDPG and uridine-diphosphate-*N*-acetylglucosamine (UDPAG) is thus confirmed, and it may therefore be regarded as 'uridine-diphosphate-glucuronic acid' (UDPglucuronic acid). Its presence in liver has been recently confirmed by Smith & Mills (1954) and by Hurlbert & Potter (1954).

Smith & Mills (1954) found that UDPglucuronic acid did not undergo the pyrophosphate fission that was observed with UDPG and UDPAG (cf. Kalckar, 1953), neither did any synthesis take place from uridine triphosphate and glucuronic acid 1-phosphate. They therefore considered that the structure of UDPglucuronic acid might not be strictly analogous to UDPG and UDPAG. Our results make this reservation unnecessary and the differences in enzymic behaviour are more probably explicable in terms of substrate specificity. We ourselves also obtained negative results in attempting to demonstrate synthesis of the factor by incubating α- or β-glucuronic acid 1-phosphate, UDP and adenosine triphosphate with liver suspensions (cf. Trucco, 1951). The possibility exists that the configuration of the glucuronic acid–phosphate bond in UDPglucuronic acid might differ from that in UDPG, which most probably is α (Paladini & Leloir, 1952). In the recently described syntheses of trehalose (Leloir & Cabib, 1953) and of sucrose (Leloir & Cardini, 1953) the UDPG gives rise to an α-glucoside. All the naturally occurring glucuronides have the β configuration, but in our present state of knowledge it is not possible to conclude that the configuration of the UDPglucuronic acid is also β, since inversion might occur during the formation of the glycosidic bond. This has been shown to take place during the phosphorolysis of maltose by certain bacterial extracts (Fitting & Doudoroff, 1952).

Although it is not certain that the glucuronyl transfer from the UDP to the acceptor represents the final stage of glucuronide synthesis *in vivo* or in liver slices, the available evidence suggests this to be true. Both UDPglucuronic acid and the enzyme system have so far been detected only in the liver (Dutton & Storey, 1954), whilst with tissue slices the liver, and to a very minor extent the kidney, were the only organs tested which showed activity (Storey, 1950). In liver slices and in suspensions the formation of glucuronides was inhibited by 'glucuronate' prepared from glucurone by treating it with a solution of sodium bicarbonate, but not when sodium hydroxide was used instead (Storey, 1950; Dutton & Storey, 1954; cf. Sie & Fishman, 1954). Previous work has also shown that β-glucuronidase is not concerned in glucuronide formation, either in the intact animal and whole-cell preparations (Levvy, 1952, 1953), or in the present system with broken-cell suspensions (Dutton & Storey, 1954).

Storey (1950) suggested that the final step of glucuronide synthesis in liver slices might be the reaction of glucuronic acid 1-phosphate with the acceptor under the influence of a phosphorylase.

However, all experiments in which the α- and β-glucuronic acid 1-phosphates were added to liver suspensions in presence of *o*-aminophenol were negative (Dutton & Storey, 1954). It is now fairly clear that the glucuronyl donor is not a simple phosphate ester but one which can be regarded as a uridyl-substituted glucuronic acid 1-phosphate. The reaction proposed represents a new type for the formation of glycosidic bonds, since unlike the well-known phosphorolytic reactions inorganic phosphate does not take part (cf. Kalckar, 1953). The syntheses of trehalose (Leloir & Cabib, 1953) and of sucrose (Leloir & Cardini, 1953) are also examples of UDP acting as a glycosyl donor. The sucrose reaction was reversible, but we have not succeeded in showing this for glucuronyl transfer. These reactions are all to be distinguished from the pyrophosphate cleavages in which uridyl transfer takes place (Kalckar, 1953, 1954).

Although Leloir and his co-workers showed originally that UDPG was the coenzyme of galactowaldenase (Caputto *et al.* 1950), the presence of the nucleotide in yeast unadapted to galactose and in animal tissues led them to conclude that this was not its only function. The isolation of an acetylaminouronic acid derivative of UDP (Park, 1952*b*), of UDPAG (Cabib *et al.* 1953), of UDPglucuronic acid (Dutton & Storey, 1953; and present paper), of guanosine-diphosphate-mannose (Cabib & Leloir, 1954) and of UDP-acetylgalactosamine (Videla, 1954), and the apparently wide distribution of at least certain of these in animal tissues (Caputto *et al.* 1950; Rutter & Hansen, 1953; Schmitz *et al.* 1954; Smith & Mills, 1954) and in plants (Buchanan *et al.* 1952) has led to speculation as to their possible role in polysaccharide synthesis. The results of Leloir & Cabib (1953), Leloir & Cardini (1953) and probably those of the present authors show that certain UDP compounds can donate glycosyl groups with the formation of a glycosidic linkage; but whether this process is extended to the synthesis of such mucopolysaccharides as hyaluronic acid, chondroitin sulphuric acid and heparin, which are built up mainly from acetylglucosamine, acetylgalactosamine and glucuronic acid units, is as yet unknown. An obvious difficulty from this view point is that UDPglucuronic acid and the conjugating enzyme system have so far not been demonstrated with certainty in any tissue other than the liver, but one possibility might be that this organ has a specific function in forming a disaccharide unit, *N*-acetylhexosamine glucuronide, as suggested by Smith & Mills (1954).

If UDPglucuronic acid is indeed concerned in the synthesis of mucopolysaccharides, then we may well inquire whether glucuronide formation does not represent a diversion from the normal course of glucuronic acid metabolism, and whether glucuronide-forming compounds (for which virtually the sole structural requirement appears to be an hydroxyl group) should not be regarded merely as trapping agents for active glucuronic acid, just as aromatic amines trap active acetyl groups by reacting with acetylcoenzyme A. It might then be largely incidental if the resulting glucuronide were less toxic or active physiologically than the parent acceptor. Ester sulphate formation might conceivably be regarded in the same light, for many hydroxyl compounds form both glucuronides and ester sulphates, and in liver slices the two processes apparently compete for the available phenol acceptor (Storey, 1950). Evidence for an active form of sulphate which will react directly with the hydroxyl compound has been presented by Bernstein & McGilvery (1952). Boström & Månsson (1953) showed that the uptake of $^{35}SO_4$ into the chondroitin sulphuric acid of cartilage slices was stimulated by a thermostable substance present in calf liver, and they suggested that it might be a cofactor for the sulphate-esterifying system. All these observations encourage the hope that further study of the sulphate-conjugation mechanism and of the reactions undergone by UDPglucuronic acid may yield considerable insight into the manner of mucopolysaccharide synthesis.

SUMMARY

1. The active liver factor responsible for glucuronide synthesis in liver suspensions has been isolated from liver extracts by ethanol fractionation of barium salts and ion-exchange chromatography, and shown to be a nucleotide containing uridylic acid, total P, labile P and glucuronic acid in the molecular proportions 1:2:1:1.

2. Hydrolysis of the nucleotide with N acid for 15 min. at 100° gave uridine 5′-phosphate, inorganic P and glucuronic acid.

3. Hydrolysis with 0·1N acid for 7 min. at 100° gave uridine 5′-pyrophosphate, uridine 5′-phosphate, glucuronic acid 1-phosphate, glucuronic acid and a trace of inorganic phosphate.

4. On the basis of the above findings it is proposed that in the nucleotide the uridine 5′-phosphate is linked to the glucuronic acid 1-phosphate through a pyrophosphate bond. It is suggested that the nucleotide be termed 'uridine-diphosphate-glucuronic acid' (UDPglucuronic acid).

5. Confirmation of this structure has been obtained from the study of the action of Russell's viper and cobra venoms.

6. The available evidence suggests that the reaction between the UDPglucuronic acid and the acceptor (ROH) is

UDPglucuronic acid + ROH →
UDP + R–O—glucuronic acid.

7. The possible relationship of this reaction to mucopolysaccharide synthesis is discussed.

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Metabolism of Thyroid Hormones

THE DEIODINATION OF THYROXINE AND TRIIODOTHYRONINE *IN VITRO*

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Recent work on the occurrence and properties of 3:5:3′-triiodo-L-thyronine (Gross & Pitt-Rivers, 1952*a*, *b*, *c*; Roche, Lissitzky & Michel, 1952) strongly suggests that this substance is the active form of the thyroid hormone. On the other hand, it is well established that the main thyroid substance circulating in the blood is thyroxine (Harington, 1944; Taurog & Chaikoff, 1948; Laidlaw, 1949; Leblond & Gross, 1949). It therefore seems necessary to postulate the conversion of thyroxine into triiodothyronine in the peripheral tissues, a process which has not been demonstrated with certainty. Earlier work in this field has recently been reviewed (Maclagan & Wilkinson, 1952; Roche & Michel, 1954) and during the preparation of this paper a more definite claim has been made by