URIDINE NUCLEOTIDE COMPOUNDS OF LIVER

by

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INTRODUCTION

In 1950 CAPUTTO, LELOIR, CARDINI AND PALADINI[1] reported the isolation from yeast of the coenzyme for the enzyme galacto-waldenase which is responsible for the conversion of galactose-1-phosphate into glucose-1-phosphate. They showed this coenzyme to be uridine-diphosphate-glucose (UDPG). PALADINI AND LELOIR[2] showed that preparations of UDPG from yeast contained uridine-5-monophosphate (UMP) and another substance similar to UDPG, which was referred to as UDPX. This latter substance has recently been identified as uridine-diphosphate-acetylglucosamine (UDPAG) (CABIB, LELOIR AND CARDINI[3]). PARK[4-6] has reported the isolation from cultures of *Staphylococcus aureus* H treated with penicillin, of three uridine pyrophosphate compounds analogous to UDPG in which the carbohydrate portion attached to the pyrophosphate is an acetylamino uronic acid, and in two of these compounds alanine, and a peptide containing alanine, lysine and glutamic acid, are bound to the carboxyl group of the acetyl amino uronic acid.

DUTTON AND STOREY[7] showed that a boiled extract of liver was a necessary addition to liver homogenates to promote glucuronide synthesis, and they isolated from liver[8] a substance, which, on the basis of analytical data, might be uridine diphosphate glucuronic acid. When incubated with liver homogenates and *o*-aminophenol or (—)menthol, they found a transfer of approximately one equivalent of glucuronic acid for each equivalent of uridylic acid present in the compound with the formation of the corresponding glucuronide.

Little is known about the function of these uridine diphosphate compounds, apart from the co-waldenase activity of UDPG. TRUCCO[9] found that the incubation of uridine diphosphate (UDP), adenosine triphosphate (ATP) and glucose-1-phosphate with a yeast extract led to the formation of UDPG. Recent work[10-11] indicates that this reaction occurs in two steps;

$$\text{UDP} + \text{ATP} \rightleftharpoons \text{UTP} + \text{ADP}$$

$$\text{UTP} + \text{glucose-1-phosphate} \rightleftharpoons \text{UDPG} + \text{pyrophosphate},$$

both reactions being reversible.

In the present work, the main interest is in the isolation and properties of the

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cofactor of glucuronide synthesis. At the same time it has been found that UDPG and UDPAG are present in liver, and some enzymic reactions involving these compounds are reported.

A preliminary report of some of this work has already appeared[12].

EXPERIMENTAL

Materials

The uridine nucleotide compounds described in the text were isolated from guinea pig liver.

α-glucuronic acid-1-phosphate was prepared by the method of MARSH[13].

β-glucuronic acid-1-phosphate was prepared by the method of TOUSTER AND REYNOLDS[14].

Triphosphopyridinenucleotide (TPN), 80% purity, was obtained from Sigma Chemical Company, St. Louis, Mo., USA.

^{32}P labelled inorganic pyrophosphate was prepared from $Na_2H^{32}PO_4$ (obtained from the Radiochemical Centre, Amersham, England) by the method of KORNBERG AND PRICER[15].

Uridine triphosphate was a synthetic preparation, prepared and kindly donated by Professor A. R. TODD, Cambridge University, and found to contain 50% UTP, 40% UDP and 10% UMP by chromatography and enzymic assay by the method of BERG AND JOKLIK[10].

Glucose-6-phosphate dehydrogenase (*Zwischen ferment*) was prepared from dried yeast (Kongens Bryghus, Copenhagen) by the method of LE PAGE AND MULLER[16]. This preparation has been found to contain the enzyme uridyl transferase[11] which catalyses the reaction UDPG + pyrophosphate $\rightleftharpoons$ UTP + glucose-1-phosphate, and the enzyme nucleoside diphosphokinase[10] which catalyses the reaction: UTP + ADP $\rightleftharpoons$ ATP + UDP. This *Zwischen ferment* preparation has been used as a source of these latter two enzymes as well as glucose-6-phosphate dehydrogenase.

Yeast hexokinase was prepared and purified by the method of BERGER, SLEIN, COLOWICK AND CORI[17].

Adenylic acid deaminase was prepared from rabbit muscle by procedure A of KALCKAR[18].

Phosphoglucomutase was prepared and purified from rabbit muscle by the method of NAJJAR[19].

UDPG and UDPAG were prepared from yeast by the method of CAPUTTO *et al.*[1] and separated by paper chromatography.

Nuclei were isolated from guinea pig liver by the isotonic sucrose method of SCHNEIDER[20]. The initial liver homogenate in ice cold 0.25 *M* sucrose was strained through nylon gauze before centrifuging. The nuclear fraction obtained at 600 g was washed five times on the centrifuge at 500–600 g with 0.25 *M* sucrose, at which time it was found to be free from cytoplasmic particles as judged microscopically. Centrifuging was carried out at 0° in an International Refrigerated Centrifuge using head No. 269.

METHODS

Paper chromatography of the nucleotides was carried out on Whatman No. 1 paper washed with 2 *N* acetic acid, using the neutral ammonium acetate — ethanol solvent of PALADINI AND LELOIR[2]. Solutions were applied to the paper from Carlsberg micro-pipettes and dried with a stream of cold air. Drying of chromatograms after development (24–40 hours) was carried out at room temperature to avoid decomposition of some of the labile nucleotide compounds. The nucleotides were localised on the paper by the method of MARKHAM AND SMITH[21] using a Mineralight lamp. Relative proportions of the nucleotides were obtained by scanning a 1 cm strip in the Beckman Spectrophotometer (Model DU) using an apparatus based on that of TENNENT, WHITLA AND FLOREY[22]. Standards were run on each chromatogram since R_F values vary considerably with temperature.

Paper chromatography of sugars was carried out with the ethyl acetate-pyridine-water solvent of JERMYN AND ISHERWOOD[23].

Ionophoresis of sugars on paper was carried out by the method of CONSDEN AND STANIER[24] using Whatman No. 1 paper and a borate buffer of pH 8.6. Sugars were located by the aniline phthalate reagent of PARTRIDGE[25] or with the modified ELSEN AND MORGAN reagent[26].

Enzymic assay of UTP was carried out by the method of BERG AND JOKLIK[10] using the enzyme nucleoside diphosphokinase which catalyses the reaction: UTP + ADP $\rightleftharpoons$ ATP + UDP, and the ATP estimated by the method of KORNBERG AND PRICER[27], the formation of reduced TPN being followed spectrophotometrically at 340 mμ.

Adenylic acid was estimated by the spectrophotometric method of KALCKAR[18] employing adenylic acid deaminase.

Pyrophosphorolysis of UDPG. In addition to the identification of this compound chromatographically, UDPG was estimated by means of the reaction[11]: UDPG + pyrophosphate $\rightleftharpoons$ UTP + glucose-1-phosphate.

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The glucose-1-phosphate was converted to glucose-6-phosphate by phosphoglucomutase and this latter substance estimated by the method of KORNBERG AND PRICER[27]. The whole reaction was carried out spectrophotometrically.

In vitro assay of glucuronide synthesis was determined by a method based on that of LEVVY AND STOREY[28] as follows: 2 ml digests containing 0.5 ml 0.2 *M* glycylglycine buffer pH 7.7 10 μ moles $MgCl_2$, 0.5 μ moles *o*-aminophenol, 0.3 ml supernatant obtained by centrifuging a 1 in 6 water homogenate of guinea pig liver at 10,000 *g* for 10 minutes, with the addition of boiled liver extract or fractionated cofactor preparation, were incubated at 37° for a specified time. The reaction was stopped and protein precipitated by the addition of 2 ml *M* glycine-trichloracetic acid buffer pH 2.2. After centrifugation, the *o*-aminophenylglucuronide was estimated in 3 ml of the supernatant by diazotisation and coupling with N-(1-naphthyl)ethylenediamine dihydrochloride. After two hours in the dark, the colours were estimated at 535 mμ (Coleman Junior Spectrophotometer), and the amount of *o*-aminophenylglucuronide determined from a calibration curve prepared from pure *o*-aminophenylglucuronide (WILLIAMS[29]).

RESULTS

Isolation of uridine nucleotides. Two methods were employed for the basic extraction of guinea pig liver: (a) ice cold trichloracetic acid extraction and (b) boiled liver extract.

Trichloracetic acid (TCA) extraction method. Guinea pig liver was homogenised (Waring Blendor) in 3 volumes ice cold 8% TCA within 1 minute of excision from animals killed by exsanguination. The homogenate was centrifuged at 0° and the residue re-extracted with an equal volume of ice cold 5% TCA and centrifuged. The combined TCA extracts were rapidly extracted three times with ether to remove the bulk of the TCA. Glycogen was precipitated by the addition of an equal volume of 96% alcohol, standing at 0° for 30 minutes and centrifuging. The supernatant was adjusted to pH 9.0 with *N* KOH, 100 ml 25% barium acetate solution added for every 100 g of original liver, followed by alcohol to a final concentration of 80% (v/v). After standing at —10° for one hour the barium salts were removed by centrifugation, washed with 80% (v/v) alcohol, and dried with absolute alcohol, ether and finally in a vacuum desiccator.

Boiled liver extract. Guinea pig liver was homogenised (Waring Blendor) in 2 vols. distilled water within 1 minute of excision from animals killed by exsanguination, and the homogenate poured into an equal volume of boiling distilled water with steam passing through in order to maintain temperature at 100°. The suspension was maintained at 100° for 1 minute and then rapidly cooled at —15° until a temperature of 0° was attained, followed by centrifugation at 20,000 *g* for 5 minutes at 0°. This solution was used as such in some experiments and is referred to as *boiled liver extract.* Glycogen was precipitated from this extract by the addition of an equal volume of 96% alcohol and 1 or 2 drops 10% perchloric acid and standing at 0° for 30 minutes followed by centrifugation. The supernatant was adjusted to pH 9.0 with *N* KOH and the barium salts precipitated and dried as for the TCA extract.

Further fractionation of total barium salts

The total barium salts from both the TCA extraction and the boiled extract of liver were fractionated in accordance with the scheme presented in Fig. 1. A temperature of 0° was maintained throughout.

Each fraction was assayed for the cofactor of glucuronide synthesis, and the result is shown in Table I.

Cofactor activity is found in the H_2O soluble, 50% EtOH precipitable barium salt fraction (B). It has been found that this fraction from a boiled liver extract is much richer in cofactor than is the same fraction from the TCA extraction method. In view

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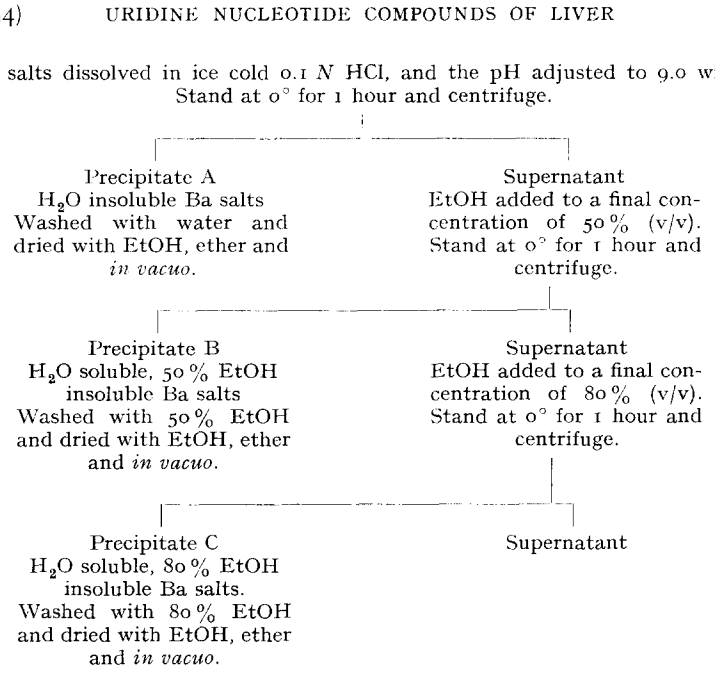

Fig. 1. Scheme of fractionation of total barium salts from guinea pig liver.

TABLE I

EXAMINATION OF THE BARIUM FRACTIONS FROM GUINEA PIG LIVER FOR THE COFACTOR OF GLUCURONIDE SYNTHESIS

30 mg each of fractions A, B and C were dissolved in water at 0° with the aid of *N* HCl, barium removed with K_2SO_4 and centrifugation, the supernatant adjusted to pH 7 and diluted to 3 ml. 0.3 ml aliquots were assayed for glucuronide synthesis cofactor.

Fraction	*o-aminophenylglucuronide formed (mμ moles)*
A	1.5
B	38.0
C	2.6

of this finding, further purification of the cofactor was confined to Fraction B from the boiled liver extract. Paper chromatographic examination of this fraction (after removal of barium) using the neutral ammonium acetate—ethanol solvent[2] gave the result shown in Fig. 2.

The individual U.V. absorbing areas of the chromatogram were eluted with the minimum volume of distilled water, and the eluate assayed for the glucuronide synthesis cofactor. It was found that cofactor activity was entirely located in area 4 of the chromatogram in Fig. 2. At the same time, each eluate was examined for glucuronic acid by the Tollens naphthoresorcinol reaction, and this substance was found to be present only in the same area as the cofactor activity.

It was observed that the R_F value of the glucuronide synthesis cofactor was almost identical with that of adenosine-5-monophosphate (AMP), and assay of the eluate of this area in 0.2 *M* succinate buffer pH 6 by the deaminase method of KALCKAR[18] indi-

cated that at least 50% of the U.V. absorbing material eluted from area 4 was AMP. AMP was removed from the water-soluble 50% EtOH precipitable barium salts by means of Dowex 50. The barium salts were dissolved in ice cold 0.1 *N* HCl and passed through a Dowex 50 H^+ column (1.5 cm × 2.6 cm^2 for approximately 20 μM material calculated on the basis of U.V. absorption for uridine compounds) at the rate of about 0.3 ml per minute, and the column washed with distilled water. Collection of effluent started when the pH fell below 3 and continued until the pH rose to 3 gain. The column was jacketed with ice and the effluent collected on ice. The eluate was neutralised to

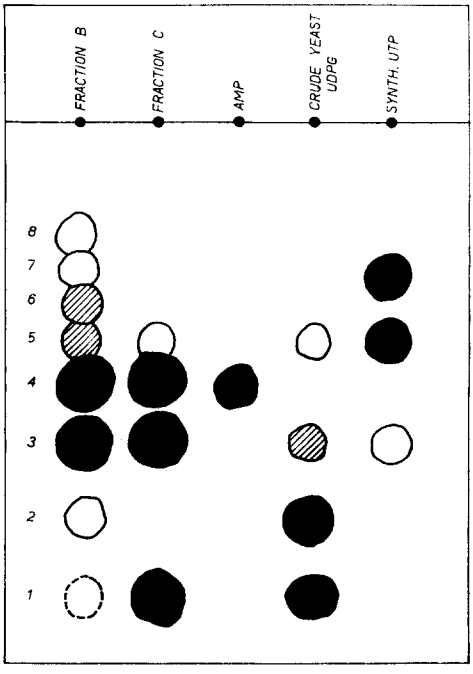

Fig. 2. Paper chromatography of Fractions B and C from guinea pig liver using Whatman No. 1 acid washed paper and ammonium acetate — ethanol solvent; 36 hours development. 1. UDPAG, 2. UDPG, 3. UMP, 4. AMP, 5. UDP, 6. ADP, 7. UTP, 8. ATP. Crude yeast UDPG preparation and synthetic UTP, UDP and UMP mixture were used as markers.

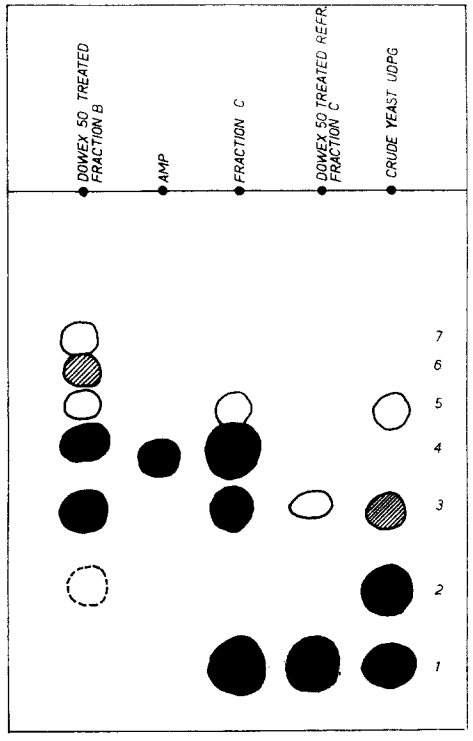

Fig. 3. The effect of Dowex 50 treatment on fractions B and C. Details as Fig. 2.

pH 7.0 and stored at −15°. Assay by the deaminase reaction[18] showed the complete absence of AMP.

Assay of the glucuronide synthesis cofactor showed that no loss occurred provided all operations were carried out at 0°. Paper chromatography of an aliquot of the effluent from the Dowex 50 H^+ column was carried out, using the neutral ammonium acetate—EtOH solvent[2], and the result is shown in Fig. 3.

The components of this chromatogram were eluted with the minimum volume of distilled water and assayed for the glucuronide synthesis factor. This substance was confined entirely to position 4. This area was also assayed for AMP by the deaminase

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reaction[18] and was found to be entirely free of this material. It was now observed that the R_F value of the cofactor was slightly less than the marker spot of AMP.

Dowex 1 fractionation of total barium salts

In an attempt to obtain a more highly purified preparation of the glucuronide synthesis cofactor, resort was made to fractionation on a Dowex 1 Cl column by the method developed by COHN[30]. 0.7 g of the total barium salts from the boiled extract of guinea pig liver were dissolved in ice cold 0.1 *N* HCl and Ba^{+2} removed with K_2SO_4 and centrifugation. The solution was adjusted to pH 9.0 with ammonia and applied to a Dowex 1 Cl′ column (5 cm × 0.7 cm^2). The column was washed with 20 ml distilled water and developed with increasing concentrations of HCl and NaCl. 10 ml fractions were collected on an automatic fraction collector. The optical density of each fraction at 260 m μ was determined in the Beckman spectrophotometer, and the results are shown in Fig. 4.

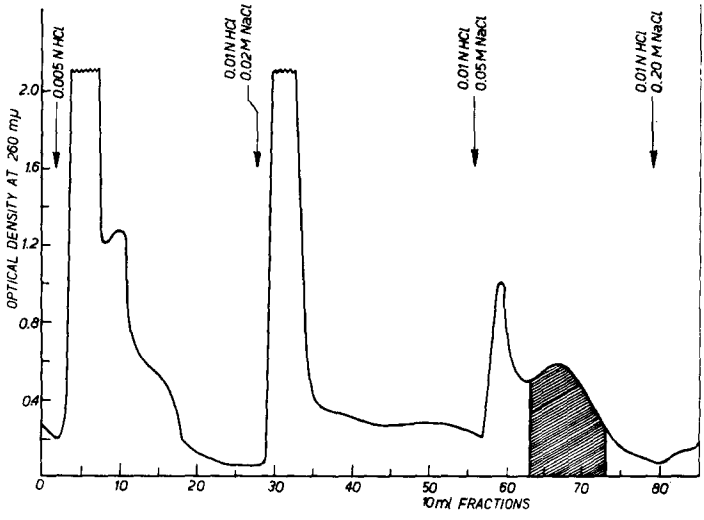

Fig. 4. Dowex 1 fractionation of total barium salts of guinea pig liver. Details in text. The shaded area indicates the position of the cofactor of glucuronide synthesis.

Each of the main peaks was assayed for glucuronide synthesis cofactor and it was found to be present in a peak between fractions 63–74. These fractions were pooled, the pH adjusted to 9 with KOH, and the barium salts precipitated with barium acetate and EtOH to 50% (v/v). After standing at —15° for one hour, the barium salts were centrifuged, washed and dried. The barium salts were dissolved in ice cold 0.01 *N* HCl, the barium removed with K_2SO_4 and the solution neutralised. This solution was assayed for glucuronide synthesis cofactor and chromatographed in the neutral ammonium acetate solvent[2]. The results indicated that it contained only 20% cofactor and that the main component was UMP. In view of the fact that free UMP is eluted from the column in 0.005 *N* HCl it is likely that the UMP in the cofactor fraction arises from breakdown of the cofactor. Consequent upon the poor yields obtained by this method, it was decided to abandon it in favour of the barium fractionation and paper chromatographic method outlined in the previous section.

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Purification of the uridine compound chromatographically identical with the UDPAG of yeast was carried out in a manner similar to the purification of the cofactor of glucuronide synthesis. The UDPAG of liver is found in the barium salts precipitated between 50–80% (v/v) ethanol (Fraction C, Fig. 1), and chromatography of this fraction after removal of barium gave the result shown in Fig. 2. It was found that the TCA extraction of liver gave higher yields of this compound than did the boiled liver extract, and the former method of extraction was therefore employed for this compound. AMP present in Fraction C of the TCA extract was removed on a Dowex 50 H^+ column as in the purification of the cofactor of glucuronide synthesis. The preparation after removal of AMP was refractionated according to Fig. 1, and fraction C from this second fractionation was chromatographed after removal of barium, giving the result shown in Fig. 3. The U.V. absorbing material consisted almost entirely of UDPAG with a small amount (approx. 5%) of UMP. Analysis of the preparation for hexose monophosphates by the method of KORNBERG AND PRICER[27], indicated that not more than 5% of these compounds are present in this material.

Properties of glucuronide synthesis cofactor and liver UDPAG

Both of these compounds prepared as above were isolated in chromatographically pure form by chromatographing a wide band (10 cm) of the solution on Whatman No. 1 paper for about 30 hours in the neutral ammonium acetate-ethanol solvent[2], and eluting the appropriate band in distilled water.

The effects of acid hydrolysis were studied, the solutions being hydrolysed at 100° for 10 mins at pH 2 (HCl or H_2SO_4) and for 90 mins at pH 1. The solutions were subsequently neutralised, concentrated and chromatographed in the neutral ammonium acetate-ethanol solvent. The resulting chromatograms were scanned for U.V. absorption in the Beckman spectrophotometer and the results are shown in Fig. 5 for the glucuronide synthesis cofactor, and in Fig. 6 for liver UDPAG.

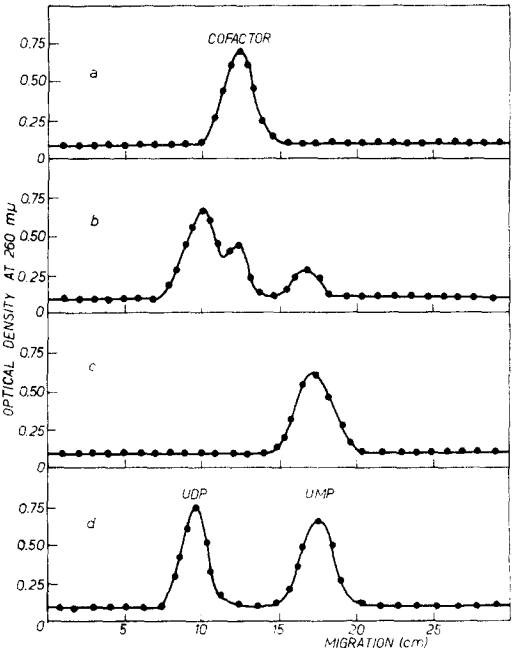

Fig. 5. Chromatograms of the hydrolysis products of the cofactor of glucuronide synthesis. Samples run simultaneously in ammonium acetate-ethanol solvent for 36 hours and scanned in the Beckman spectrophotometer at 260 mμ.
a. Glucuronide synthesis cofactor;
b. the same after heating at 100° for 10 mins at pH 2;
c. heated at 100° for 90 mins at pH 1;
d. UDP and UMP markers.

Hydrolysis of the glucuronide synthesis cofactor for 10 mins at pH 2 and 100° caused a complete loss of activity in the glucuronide synthesis system and the release of a free reducing carbohydrate which was identified by ionophoresis on paper[24] as glucuronic acid. No other free carbohydrate was produced by these conditions of hydrolysis.

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Under the same conditions of hydrolysis of the UDPAG of liver, the presence of free N-acetyl glucosamine was revealed by the reaction of AMINOFF, MORGAN AND WATKINS[21], and was confirmed by ionophoresis on paper[24] and by paper chromatography in ethyl acetate-pyridine-water[23].

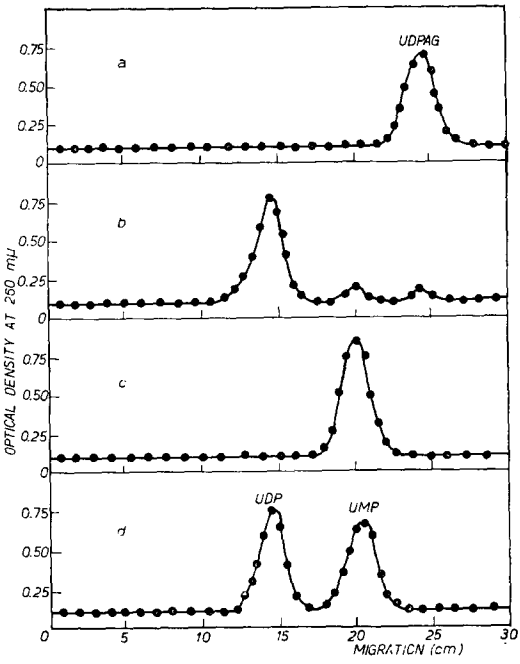

Fig. 6. Chromatograms of the hydrolysis products of liver UDPAG. Samples run simultaneously in ammonium acetate-ethanol solvent for 36 hours and scanned in the Beckman spectrophotometer at 260 mμ.
a. Liver UDPAG;
b. the same heated at 100° for 10 mins at pH 2;
c. heated at 100° for 90 mins at pH 1;
d. UDP and UMP markers.

The U.V. absorption spectra of the glucuronide synthesis cofactor and liver UDPAG were typical for uridine compounds, showing a $\lambda_{max.}$ at 262 mμ and a $\lambda_{min.}$ at 232, with an E_{262}/E_{232} ratio of 4. The spectra were completely abolished by treatment with bromine water[1].

Other uridine compounds of liver

CAPUTTO *et al.*[1] and RUTTER AND HANSEN[32] have reported the presence of UDPG in liver, using enzymic methods of assay. In fraction B (Fig. 1) of boiled extracts of liver, a chromatographic component was frequently observed, with the same R_F value as the UDPG of yeast (Fig. 2). This substance was eluted from a paper chromatogram, and showed a typical uridine spectrum. It was found to participate in the pyrophosphorolysis reaction[11],

$$\text{UDPG} + \text{pyrophosphate} \rightleftharpoons \text{UTP} + \text{glucose-1-phosphate}$$

and would therefore appear to be identical with the UDPG of yeast.

Uridine-5-monophosphate (UMP) and uridine diphosphate (UDP) have been consistently found in liver extracts prepared either by extraction with TCA or boiling water. The former was identified by its U.V. absorption spectrum which showed a maximum at 262 mμ (abolished by treatment with bromine water[1]). Chromatographically, this component behaved in an identical manner to uridine-5-monophosphate (Nutritional Biochemicals). UDP was identified by its uridine spectrum, by hydrolysis to UMP (90 minutes at pH 1 and 100°), and also by phosphorylation to UTP as shown in Fig. 7.

The spot corresponding chromatographically to UTP was eluted and further identified by the method of BERG AND JOKLIK[10]. This conversion of UDP to UTP confirms the finding of KORNBERG[33] who reported that UDP was phosphorylated to UTP by phosphopyruvate and pyruvatephosphokinase.

A chromatographic component with the same R_F value as UTP was frequently found in Fraction B of the boiled liver extract. This compound was eluted and found to have a uridine spectrum. It was also shown to be a nucleoside triphosphate by the method of BERG AND JOKLIK[10]. It appears probable that this substance is UTP, but insufficient material was present for a complete analysis.

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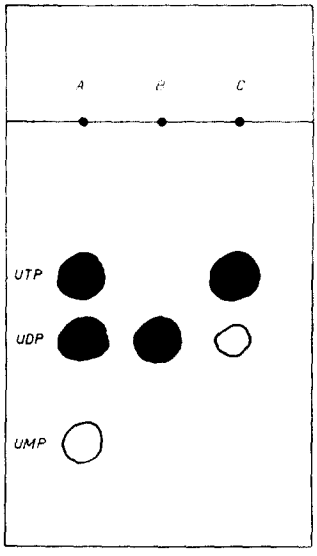

Fig. 7. The phosphorylation of UDP to UTP. 0.5 μ mole UDP (eluted from a chromatogram) incubated with 1.0 μ mole phosphoglycerate, 5 μ moles $MgCl_2$, 5 μ moles KCl, 200 μl dialysed water extract of rabbit muscle (equivalent to 60 mg original muscle) and 0.2 *M* TRIS buffer, pH 6.8, to a final volume of 0.5 ml for 30 mins at 25°. Reaction stopped and protein removed with 50 μl 10% perchloric acid and centrifugation. Nucleotides adsorbed on 10 mg acid washed charcoal for 10 mins, and eluted from the charcoal after removal of the reaction medium with 1 ml 50% ethanol for 1 hour. The eluate concentrated by an air stream at room temperature and chromatographed in ammonium acetate-ethanol for 36 hours.

A. synthetic UTP, UDP and UMP mixture;
B. UDP;
C. concentrate from reaction.

Enzymic studies on UDPAG

It was shown by MUNCH-PETERSEN *et al.*[11] that UDPG undergoes pyrophosphorylation in the presence of a yeast enzyme to produce UTP and glucose-1-phosphate. Attempts to carry out the analogous reaction with liver UDPAG failed to show any production of UTP as determined by chromatography or by the method of BERG AND JOKLIK[10]. Since it was shown by HOGEBOOM AND SCHNEIDER[34] that the enzyme responsible for the reaction:

ATP + nicotinamide mononucleotide $\rightleftharpoons$ diphosphopyridinenucleotide + pyrophosphate

is present in isolated rat liver nuclei, it was decided to investigate liver nuclei as a source of an enzyme which would pyrophosphorylate UDPG and UDPAG.

UDPG, prepared from yeast and purified by paper chromatography, was incubated with a suspension of guinea pig nuclei and inorganic pyrophosphate in Tris (hydroxymethyl) amino methane buffer (TRIS) pH 7.8, and the result is shown in Fig. 8.

The chromatographic area corresponding to UTP was eluted, and the identity of this substance confirmed by the assay method of BERG AND JOKLIK[10]. A large production of UDP and UMP was also observed in this experiment.

The analogous reaction with UDPAG isolated from liver was carried out, using pyrophosphate labelled with ^{32}P. The chromatogram of the reaction products was scanned for U.V. absorption in the Beckman spectrophotometer. The 1 cm wide strips

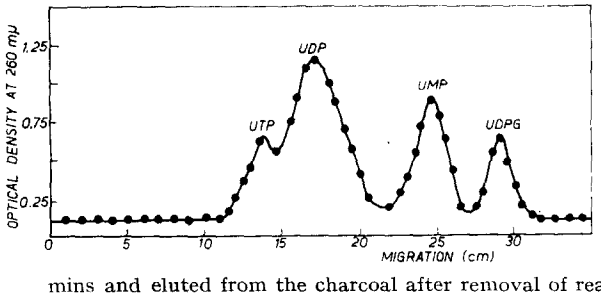

Fig. 8. The pyrophosphorolysis of UDPG by isolated guinea pig liver nuclei. 2 μ moles UDPG (yeast) incubated with 5 μ moles $MgCl_2$, 10 μ moles potassium pyrophosphate, 400 μl nuclei suspension in 0.25 *M* sucrose (equivalent to 100 mg original liver) and 0.2 *M* TRIS buffer, pH 7.8, to a final volume of 1 ml for 30 mins at 25°. Reaction stopped and protein removed with 100 μl 10% perchloric acid and centrifugation. Nucleotides adsorbed on 50 mg acid washed charcoal for 10 mins and eluted from the charcoal after removal of reaction medium with 2 ml 50% ethanol for 1 hour. The eluate concentrated by an air stream at room temperature, and chromatographed in ammonium acetate-ethanol for 40 hours. Chromatogram scanned in Beckman spectrophotometer at 260 mμ. Positions of spots identified by markers run simultaneously.

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were further scanned for radioactivity using an endwindow Geiger-Müller tube with a lead foil mask having a 2 mm wide slit. The Geiger tube was attached to a standard scaling unit (Bruel & Kjaer, København). The results are shown in Fig. 9. In this experiment the effect of nuclei alone on UDPAG was studied, and also the effect of fluoride ions on the breakdown of UTP and UDPAG by reactions other than pyrophosphorolysis.

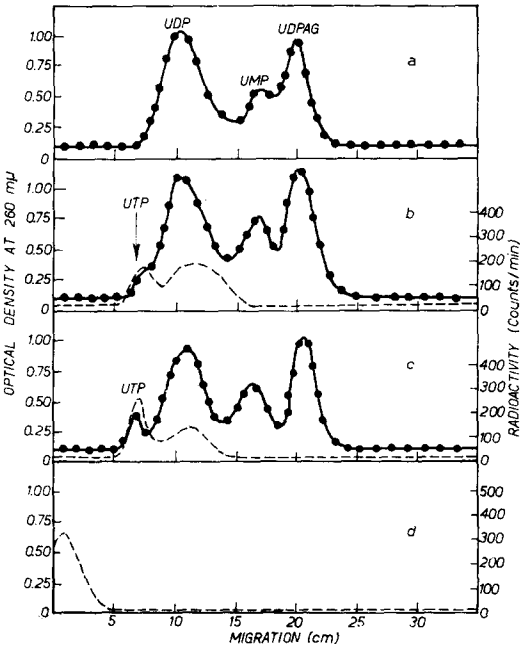

Fig. 9. The pyrophosphorylation of liver UDPAG by isolated guinea pig liver nuclei. (a). 2 μ moles UDPAG incubated with 5 μ moles $MgCl_2$, 400 μl nuclei suspension in 0.25 M sucrose (equivalent to 300 mg original liver) and 0.2 M TRIS buffer pH 7.8 to a final volume of 3 ml for 30 mins at 25°. (b). The same as (a) with the addition of 2 μ moles inorganic pyrophosphate labelled with ^{32}P (1 μc). (c). The same as (b) with the addition of 60 μ moles NaF. Reaction mixtures treated as in Fig. 8. Chromatogram developed for 24 hours with the ammonium acetate-ethanol solvent. Position of spots identified by markers run simultaneously. (d). Inorganic pyrophosphate marked with ^{32}P chromatographed without treatment. Chromatograms scanned in Beckman spectrophotometer at 260 mμ and for radioactivity. In experiments (b) and (c) the charcoal used was also washed with inorganic phosphate and pyrophosphate.

It will be seen that there is a definite production of radioactive UTP, the identity of which was further confirmed by enzyme assay[10]. In addition however, there is considerable radioactivity in the UDP area indicating a breakdown of UTP to UDP. 0.02 M fluoride causes some inhibition of this breakdown of UTP (Fig. 9(c)). It is clear from Fig. 9(a) that nuclei alone cause considerable breakdown of UDPAG to UDP and UMP; some free uridine was also observed in this experiment (not shown in Fig. 9).

The granules and supernatant from the centrifugation of the isotonic sucrose homogenate of liver[20] appeared to be inactive in the pyrophosphorolysis of UDPG and UDPAG.

Enzymic studies on the cofactor of glucuronide synthesis

Since the analytical data would appear to indicate that this compound is uridine diphosphate glucuronic acid, attempts were made to pyrophosphorylate this compound in a manner analogous to UDPG and UDPAG. These attempts were uniformly negative whether a yeast preparation or liver nuclei were used as source of enzyme. Using chromatographically purified cofactor one typical experiment using liver nuclei and ^{32}P-pyrophosphate is recorded in Fig. 10.

The only U.V. absorbing material produced in this experiment was UMP, with no formation of either UTP or UDP. An unexplained finding was the presence of a slow moving radioactive spot which showed no U.V. absorption. The granules and supernatant were also devoid of pyrophosphorolytic activity.

The demonstration by MUNCH-PETERSEN *et al.*[11] of the reversibility of the pyrophosphorolytic split of UDPG, suggested the possibility of an enzymic synthesis of uridine diphosphate glucuronic acid (UDPGA) by the reaction:

$$\text{UTP} + \text{glucuronic acid-1-phosphate} \rightleftharpoons \text{UDPGA} + \text{pyrophosphate}$$

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It was found, in the present work, that the reaction with glucose-1-phosphate and UTP was specific for the α-compound, the β-compound giving no formation of UDPG.

Experiments in which UTP was incubated with either α or β glucuronic acid-1-phosphate, and either a yeast preparation or liver nuclei as source of enzyme, with the addition of inorganic pyrophosphatase[35] to remove any pyrophosphate formed, showed no production of the cofactor of glucuronide synthesis either by chromatography or by *in vitro* synthesis of *o*-aminophenylglucuronide.

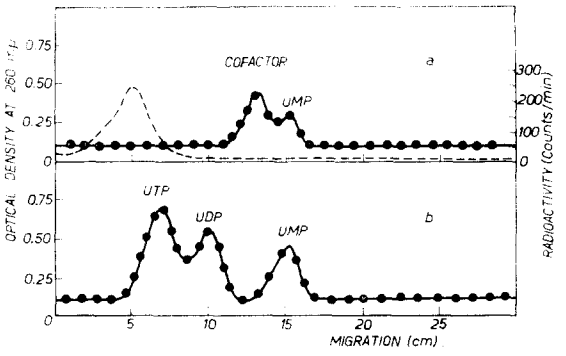

Fig. 10. The effect of isolated guinea pig liver nuclei and pyrophosphate on the cofactor of glucuronide synthesis. (a). 0.25 μ moles cofactor incubated with 5 μ moles $MgCl_2$, 2 μ moles inorganic pyrophosphate labelled with ^{32}P (1 μc), 250 μl nuclei suspension in 0.25 *M* sucrose (equivalent to 200 mg original liver) and 0.2 *M* TRIS buffer pH 7.8 to a final volume of 2 ml, for 30 mins at 25°. Reaction mixture treated as in Fig. 8 using 5 mg acid washed charcoal, which was also washed with inorganic phosphate and pyrophosphate. (b). Marker spots of UTP, UDP and UMP. Chromatogram developed for 24 hours with the ammonium acetate-ethanol solvent and scanned in the Beckman spectrophotometer at 260 mμ and also for radioactivity.

Investigations have been carried out on the transfer reaction between the cofactor and *o*-aminophenol. This reaction was found to proceed just as efficiently in glycylglycine or TRIS buffers as in phosphate buffers, and showed a broad pH optimum around pH 7.7. It was found that water homogenates of liver were as efficient a source of conjugating enzyme as KCl homogenates, and that the activity of this enzyme was undiminished in the supernatant from a water homogenate centrifuged at 10,000 *g* for 10 minutes. Storage of homogenates or supernatants for 2 days at 0° or — 15° caused complete loss of activity.

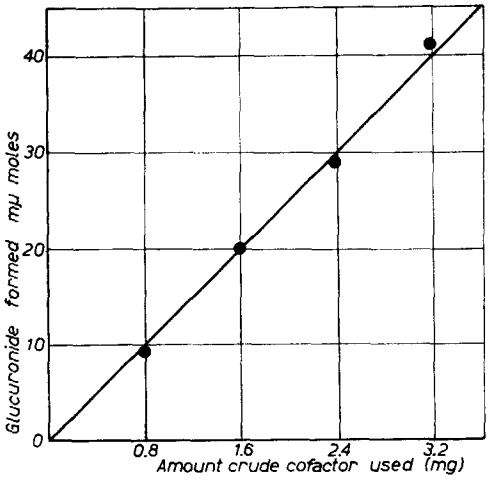

Fig. 11. The relation of *o*-aminophenylglucuronide formed to the amount of glucuronide synthesis cofactor used.

The conjugation reaction was found to be very rapid, and using the technique described in the methods section, the reaction was complete in 10–15 minutes.

There was found to be a straight line relationship between the amount of *o*-aminophenylglucuronide formed and the amount of cofactor used, and such an experiment, using a crude cofactor preparation, is shown in Fig. 11.

In the case of the chromatographically purified cofactor, it was found that the amount of glucuronide formed was equivalent on a molar basis, to the amount of cofactor used (calculated on the basis of a molar extinction of 10^4 for the cofactor). The concentration of *o*-aminophenol used made little difference, provided sufficient *o*-aminophenol was present for complete conjugation of the amount of cofactor used.

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Using the complete liver homogenate, attempts were made to replace the cofactor by various substances, but in no case was any stimulation of glucuronide synthesis obtained. The additions used were: ATP, UTP, α- or β-glucuronic acid-1-phosphate, glucuronate, UDPG and various combinations of these substances.

Fluoride (20 mM), iodoacetate (2 mM) and cyanide (1 mM) were found to have a negligible effect on the reaction, while Mg^{+2} ions (5 mM) were found to cause some stimulation.

DISCUSSION

The presence of UDPG in liver has been demonstrated by the use of enzymic techniques[1, 32] and the isolation of a cofactor of glucuronide synthesis from liver has been reported[8], which, on analysis appears to be UDPGA. Prior to these reports, no information was available concerning the presence of free uridine nucleotides in liver tissue. In the present work, evidence has been presented for the existence in liver of UMP, UDP, UTP, UDPG, UDPAG, and a uridine diphosphate compound of glucuronic acid which is the cofactor of glucuronide synthesis. Considerable amounts of these compounds exist in liver, and it has been possible to isolate approximately 15 μM UDPAG per 100 g liver. Initial assay of a boiled extract of liver shows the presence of approximately 16 μM cofactor per 100 g liver. Considerable losses occur during isolation however, and it has not been possible to recover more than approximately 25% of this amount. Significant amounts of UMP and UDP are also present in liver, but the quantity of UTP extracted appears to be small. Only the water-soluble barium salts have so far been examined in any detail, however, and it is quite probable that UTP will occur in larger amounts in the water-insoluble barium salts. Examination of this fraction will be the subject of a further communication.

The great loss of the glucuronide synthesis cofactor during purification on the Dowex Cl′ column, indicates the unstable nature of this compound. It is very probable that the use of Dowex 1 formate columns and formate buffers of a higher pH will enable a simpler preparation with larger yields to be devised. This problem is also at present under investigation.

Chemical studies on the UDPAG of liver make it fairly certain that this material is identical with the UDPAG of yeast[3]. The fact that liver UDPAG undergoes a pyrophosphorolytic split to give UTP is further evidence for the structure of this compound. It is a significant finding that the pyrophosphorolysis of UDPAG occurs only with liver nuclei preparations, and not with yeast preparations which rapidly pyrophosphorylate UDPG. The results shown in Fig. 9 indicate that the UTP formed in the pyrophosphorolysis of UDPAG is broken down to UDP, as indicated by the formation of radioactive UDP. This breakdown of UTP appears to be inhibited somewhat by fluoride ions (0.02 M). In addition, however, UDPAG is degraded by nuclei preparations to give non-radioactive UDP, UMP and free uridine. A similar pattern is observed when UDPG is incubated with liver nuclei preparations and pyrophosphate; in this case, however, the formation of UTP is greater than that obtained by corresponding pyrophosphorolysis of UDPAG.

Chemical analysis of the glucuronide synthesis cofactor would appear to indicate a structure analogous to that of UDPG and UDPAG. When one turns to the enzymic experiments, however, certain anomalies are found. The cofactor will not undergo pyro-

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phosphorolysis with either yeast or liver nuclei preparations. The only products of reaction with liver nuclei are UMP and uridine, with no evidence for the formation of either UTP or UDP. In addition, attempts to synthesise the cofactor by means of the reaction analogous to the synthesis of UDPG[11], namely UTP + glucose 1 phosphate $\rightleftharpoons$ $\rightleftharpoons$UDPG + pyrophosphate, were completely negative. When UTP and either α- or β-glucuronic acid-1-phosphate were incubated with either a yeast preparation or liver nuclei as source of enzyme, no cofactor was formed. There are two possible interpretations of these findings. While chemical analysis suggests that the structure of the cofactor is a compound of uridine diphosphate and glucuronic acid, its exact structure may be intrinsically different from that of UDPG or UDPAG; alternatively, the enzyme systems acting upon the cofactor may be distinct from those acting upon UDPG and UDPAG.

The rapidity of the reaction for the synthesis of glucuronides from cofactor, coupled with the ratio of unity for the molar amount of glucuronide formed from cofactor and the fact that the *o*-aminophenol concentration has little effect on the reaction, suggests that the overall reaction is a simple transference of glucuronic acid to the phenol with the formation of UDP. A study of this reaction and the end products other than *o*-aminophenylglucuronide might provide some information on the structure of the cofactor.

When one turns to the possible role of these uridine compounds in liver, one enters the field of pure speculation. It has already been shown that UDPG is the coenzyme for the reaction: galactose-1-phosphate $\rightleftharpoons$ glucose-1-phosphate in yeast[1] and bacteria[32], and it is probable that this reaction also occurs in liver. BUCHANAN *et al.*[36] have also suggested that a possible role of UDPG is the transference of glucose to form disaccharides. The part played by the cofactor of glucuronide synthesis gives some indication of the possible function of UDPAG. It is quite possible that both of these compounds act as carbohydrate donors in the formation of the disaccharide unit of hyaluronic acid—namely N-acetylglucosamine-glucuronide.

The presence of other uridine compounds in liver (UMP, UDP and UTP) suggests that these compounds might well fulfil similar roles to the corresponding adenine compounds. As yet no system has been found for the phosphorylation of UMP to UDP but further work is in progress on this problem.

A preliminary examination of the water-insoluble barium salts of liver indicates that nucleoside triphosphates other than ATP and UTP may be present. If this should prove to be the case, it lends some support to the suggestion of KALCKAR[37]of the role of nucleoside triphosphates in ribonucleic acid synthesis.

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References p. 399/400.

SUMMARY

1. A cofactor of glucuronide synthesis, which appears to be a compound of uridine diphosphate and glucuronic acid, has been isolated from liver. In addition uridine diphosphate acetyl-glucosamine (UDPAG) has been isolated from liver and has been shown to be identical with the UDPAG of yeast.

2. Uridine monophosphate (UMP), uridine diphosphate (UDP), uridine triphosphate (UTP) and uridine diphosphate glucose (UDPG) have also been shown to be present in liver.

3. It has been shown that UDPAG and UDPG undergo a pyrophosphorolytic split to give UTP in the presence of isolated liver nuclei. In addition there are enzymes present in liver nuclei which cause a breakdown of UTP to UDP, and of UDPAG to UDP, UMP and uridine.

4. In view of the enzymic inactivity of the cofactor of glucuronide synthesis, it is suggested that the structure of this compound may be different from that of UDPG and UDPAG.

5. The possible function of these uridine compounds in liver is discussed.

RÉSUMÉ

1. Un cofacteur de la synthèse des glucuronides, qui semble être composé d'uridine diphosphate et d'acide glucuronique a été isolé du foie. Uridine diphosphate acetylglucosamine (UDPAG) a été isolé du foie de plus, et c'est identique avec l'UDPAG de la levure.

2. Uridine monophosphate (UMP), uridine diphosphate (UDP), uridine triphosphate (UTP) et uridine diphosphate glucose (UDPG) ont été demontrés dans la foie.

3. C'est demontré que UDPAG et UDPG souffrent une scission pyrophosphorolytique avec de noyaux isolés du foie. De plus, il y en a d'enzymes de noyaux du foie, qui peuvent scinder UTP à UDP, et UDPAG à UDP, UMP et uridine.

4. À cause de la inactivité enzymique du cofacteur de la synthèse des glucuronides, c'est possible que la structure de ce composé est différent de la structure d'UDPG et d'UDPAG.

5. Le rôle possible de ces composés du foie est discuté.

ZUSAMMENFASSUNG

1. Ein Cofaktor zur Synthese von Glucuroniden, der anscheinend eine Verbindung von Uridindiphosphat und Glucuronsäure ist, wurde aus Leber isoliert. Ausserdem wurde Uridindiphosphat-Acetylglucosamine (UDPAG) aus Leber isoliert, und es wurde bewiesen, dass diese Verbindung mit dem UDPAG der Hefe identisch ist.

2. Es wurde weiter bewiesen, dass Uridinmonophosphat (UMP), Uridindiphosphat (UDP), Uridintriphosphat (UTP) und Uridindiphosphat-Glucose (UDPG) in Leber vorhanden sind.

3. Es wurde gefunden, dass UDPAG und UDPG in Gegenwart von isolierten Leberzellkernen einem pyrophosphorolytischen Zerfall unterliegen und UTP geben. Ausserdem gibt es Fermente in Leberzellkernen, welche den Zerfall des UTP zu UDP und des UDPAG zu UDP, UMP und Uridin verursachen.

4. Wenn man die enzymatische Wirkungslosigkeit des Cofaktors der Synthese von Glucuronide in Betracht zieht, ist anzunehmen, dass die Struktur dieser Verbindung verschieden ist von der des UDPG und des UDPAG.

5. Die mögliche Wirkung dieser Uridinverbindungen in Leber wird besprochen.

REFERENCES

1 R. CAPUTTO, L. F. LELOIR, C. E. CARDINI AND A. C. PALADINI, *J. Biol. Chem.*, 184 (1950) 333.
2 A. C. PALADINI AND L. F. LELOIR, *Biochem. J.*, 51 (1952) 426.
3 E. CABIB, L. F. LELOIR AND C. E. CARDINI, *J. Biol. Chem.*, 203 (1953) 1055.
4 J. T. PARK, *J. Biol. Chem.*, 194 (1952) 877.
5 J. T. PARK, *J. Biol. Chem.*, 194 (1952) 885.
6 J. T. PARK, *J. Biol. Chem.*, 194 (1952) 897.
7 G. J. DUTTON AND I. D. E. STOREY, *Biochem. J.*, 48 (1951) xxix.
8 G. J. DUTTON AND I. D. E. STOREY, *Biochem. J.*, 53 (1953) xxxvii.
9 R. E. TRUCCO, *Arch. Biochem.*, 34 (1951) 482.
10 P. BERG AND W. K. JOKLIK, *Nature*, 172 (1953) 1008.
11 A. MUNCH-PETERSEN, H. M. KALCKAR, E. CUTOLO AND E. E. B. SMITH, *Nature*, 172 (1953) 1036.
12 E. E. B. SMITH, A. MUNCH-PETERSEN AND G. T. MILLS, *Nature*, 172 (1953) 1038.
13 C. A. MARSH, *J. Chem. Soc.*, (1952) 1578.

[14] O. TOUSTER AND V. H. REYNOLDS, *J. Biol. Chem.*, 197 (1952) 863.
[15] A. KORNBERG AND W. E. PRICER, *J. Biol. Chem.*, 191 (1951) 535.
[16] G. A. LEPAGE AND C. G. MULLER, *J. Biol. Chem.*, 180 (1949) 979.
[17] L. BERGER, M. W. SLEIN, S. P. COLOWICK AND C. F. CORI, *J. Gen. Physiol.*, 29 (1946) 379.
[18] H. M. KALCKAR, *J. Biol. Chem.*, 167 (1947) 461.
[19] V. A. NAJJAR, *J. Biol. Chem.*, 175 (1948) 281.
[20] W. C. SCHNEIDER, *J. Biol. Chem.*, 176 (1948) 259.
[21] R. MARKHAM AND J. D. SMITH, *Biochem. J.*, 45 (1949) 294.
[22] D. M. TENNENT, J. B. WHITLA AND K. FLOREY, *Anal. Chem.*, 23 (1951) 1748.
[23] M. A. JERMYN AND F. A. ISEHRWOOD, *Biochem. J.*, 44 (1949) 402.
[24] R. CONSDEN AND W. M. STANIER, *Nature*, 169 (1952) 783.
[25] S. M. PARTRIDGE, *Nature*, 164 (1949) 443.
[26] S. M. PARTRIDGE, *Biochem. J.*, 42 (1948) 238.
[27] A. KORNBERG AND W. E. PRICER, *J. Biol. Chem.*, 193 (1951) 481.
[28] G. A. LEVVY AND I. D. E. STOREY, *Biochem. J.*, 44 (1949) 295.
[29] R. T. WILLIAMS, *Biochem. J.*, 37 (1943) 329.
[30] W. E. COHN, *J. Cell. Comp. Physiol.*, 38 (1951) Suppl. 1, p. 21.
[31] D. AMINOFF, W. T. J. MORGAN AND W. M. WATKINS, *Biochem. J.*, 51 (1952) 379.
[32] W. J. RUTTER AND R. G. HANSEN, *J. Biol. Chem.*, 202 (1953) 323.
[33] A. KORNBERG, *Symposium on Phosphorus Metabolism*, Johns Hopkins Press, Baltimore, Vol. 1 (1951) 411.
[34] G. H. HOGEBOOM AND W. C. SCHNEIDER, *J. Biol. Chem.*, 197 (1952) 611.
[35] L. A. HEPPEL AND R. J. HILMOE, *J. Biol. Chem.*, 192 (1951) 87.
[36] J. G. BUCHANAN, J. A. BASSHAM, A. A. BENSON, D. F. BRADLEY, M. CALVIN, L. L. DAUS, M. GOODMAN, P. M. HAYES, V. H. LYNCH, L. T. NORRIS AND A. T. WILSON, *Symposium on Phosphorus Metabolism*, Johns Hopkins Press, Baltimore, Vol. 2 (1952) 440.
[37] H. M. KALCKAR, *Pubbl. Staz. Zool. Napoli*, XXIII (1951) Suppl., p. 87.

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Note added in proof

Since the completion of this work, Dr. R. B. HURLBERT, University of Wisconsin, U.S.A., has kindly informed us that he and his colleagues have independently confirmed the presence of UMP, UDP, UTP, UDPG, UDPAG and UDPGA in rat liver tissue.