indolylacetic acid. Riboflavin deficiency frequently had the same effect.

3. Mepacrine did not affect formation of the acid in the supernatant fraction of homogenized guinea-pig liver but it decreased the disappearance of the amine along other pathways.

4. Riboflavin deficiency resulted in decreased disappearance of the amine in supernatants of rat liver but not in the particulate fractions.

5. A hypothesis has been proposed that in mammalian tissue there is a pathway of metabolism of 5-hydroxytryptamine, other than the one catalysed by monoamine oxidase, which is sensitive to flavoenzyme inhibition and to riboflavin deficiency.

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Evidence for the Occurrence of Uridine Diphosphate N-Acetylglucosamine in Crustacean Tissues

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The polysaccharide chitin, which, from available evidence, is believed to consist of unbranched chains of N-acetylglucosamine residues, is a prominent constituent of the arthropod exoskeleton. Although information about chitin biosynthesis in arthropods is very sparse, its formation in *Neurospora crassa* involves uridine diphosphate N-acetylglucosamine as a glycosyl donor (Glaser & Brown, 1957). Although this and other uridine coenzymes are utilized extensively in the tissues of higher animals, relatively little is known of their biochemistry in invertebrates. This paper describes an investigation of the nucleotide content of the hypodermis and hepatopancreas of three species of Crustacea, with particular reference to the possible function of uridine diphosphate derivatives in polysaccharide biosynthesis.

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MATERIALS AND METHODS

Animals. Live specimens of the shore crab *Carcinus maenas* and of the spider crab *Maia squinado* were obtained from the Marine Biological Laboratory, Plymouth. Live lobsters (*Homarus vulgaris*) were obtained from local dealers.

Extraction of nucleotides from hepatopancreas and hypodermal tissue. (i) Perchloric acid method. The freshly excised tissue was weighed, frozen in liquid oxygen, powdered in a cold mortar and then dispersed in 2 vol. of cold 0.6N-perchloric acid. The insoluble residue was collected by filtration and re-extracted with cold 0.6N-perchloric acid. The combined filtrates were neutralized with 5N-KOH and the precipitated KClO₄ was removed by centrifuging, the neutralized extract being diluted to 5 ml. final volume for every gram of tissue used.

(ii) Aqueous extraction. The tissue was cooled on ice, weighed and minced in a chilled blender (a MSE Nelco 10 homogenizer or a Waring Blendor was used). The suspen-

sion was boiled for 2 min. and, after filtration, the residue was re-extracted with boiling water. The combined filtrates were centrifuged and had a final volume of 10–20 ml./g. of tissue.

Phenol treatment of extracts. Coloured matter in *Carcinus* extracts was removed by phenol extraction by the procedure of Storey & Dutton (1955).

Precipitation of barium salts of nucleotides. An extract was freed of glycogen by addition of an equal volume of ethanol and centrifuging. Nucleotides were precipitated from the supernatant by addition of a 20% (w/v) solution of $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.5 ml./2.5 ml. of extract) followed by 4 vol. of ethanol. The solution was adjusted to pH 9 and cooled to -14° ; the barium salts were collected by centrifuging, dissolved in cold 0.1 N-HCl and re-precipitated from cold, alkaline, 80% ethanol as before. Barium was removed by dissolving the final precipitate in the minimum quantity of cold 0.1 N-HCl and treating the solution either with Amberlite IR-100 (K^+), or with saturated K_2SO_4 solution and centrifuging; if necessary, the final solution of nucleotides was neutralized with 5 N-KOH.

Ion-exchange chromatography on Dowex 1. Dowex 1 X2, 200–400 mesh, was used for all experiments. Nucleotide mixtures were fractionated with the acid and neutral formate gradient systems of Hurlbert, Schmitz, Brum & van Potter (1954), and by stepwise elution from chloride columns (Cabib, Leloir & Cardini, 1953). Mixtures containing 5–10 μ moles of total nucleotides were resolved on columns 0.5 cm. diam. $\times$ 15 cm. high; for gradient elution with the formate systems, the formate solution passed from a reservoir to the column through an airtight system of two flasks in series, each containing 100 ml. of water initially, and magnetically stirred; 5 ml. fractions were collected. The gradient produced by systems of this type is described by Drake (1955). Mixtures containing larger amounts of total nucleotides were fractionated on columns 0.9 cm. diam. $\times$ 10–30 cm. high. Formate columns were run in the cold room (4°) and chloride columns at room temperature.

Nucleotides in formate-column effluents were rendered salt-free by concentration on a rotary evaporator, followed by freeze-drying (0.1–0.5 mm. Hg) at room temperature to remove formic acid and ammonium formate. Solutions of nucleotides containing chloride were concentrated and made salt-free by adsorbing the nucleotides on to unground Nuchar C charcoal followed by elution with 50% ethanol containing 1–2% (v/v) of NH_3 soln. (sp.gr. 0.880) (Cabib *et al.* 1953). Ammonia and ethanol were removed on a rotary evaporator. In some cases this charcoal treatment was applied to original tissue extracts to remove pigments.

For identification of nucleotides in column effluents, the pooled fractions corresponding to a given peak were desalted, concentrated and, in some cases, further purified by paper chromatography, with the neutral ammonium acetate-ethanol solvent of Paladini & Leloir (1952). Portions of the concentrated fractions were hydrolysed with 2 N-HCl in sealed tubes at 100° for 6 hr. and the free bases liberated were identified by paper chromatography in propan-2-ol-HCl-water (Wyatt, 1951), with authentic purines and pyrimidines as markers. The ratio total phosphate/base was also measured.

Compounds separated on Dowex-1 formate columns were identified by measurement of the ratio E_{275}/E_{260} and by reference to data for calibrated columns (Hurlbert *et al.*

1954). In preliminary formate separations, and with chloride-column fractionations, further confirmation was obtained by identification of the free bases and measurement of the total phosphate/base ratio.

Phosphate estimations. The methods of Chen, Toribara & Warner (1956) and of Berenblum & Chain (1938), with the modification of Bartley (1953), were employed. For estimation of total phosphate, samples were concentrated and ashed by the method of Burton & Petersen (1960). 'Labile phosphate' corresponded to that released by N-HCl at 100° in 20 min.

N-Acetylaminosugar. This was estimated by the borate method of Reissig, Strominger & Leloir (1955), and by the carbonate method of Aminoff, Morgan & Watkins (1952).

Hydroxamate tests. The following modification of the method of Peel (1951) was used. Immediately before use, an alkaline solution of hydroxylamine was prepared by mixing equal volumes of 2.5 N-NaOH and 2 M-hydroxylamine-HCl. To the sample contained in 0.5 ml. of water was added 1 ml. of alkaline hydroxylamine solution; after 15 min. at room temperature or at 37° , 0.25 ml. of 5.6 N-HCl, 0.25 ml. of FeCl_3 solution (15%, w/v, of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 0.2 N-HCl) and 1.0 ml. of water were added. The solution was mixed thoroughly and the extinction at 530 μ was measured against a 'blank' containing no ester. With ethyl acetate, a linear increase in extinction was obtained in the range 0.1–1.0 μ mole.

Prostatic phosphomonoesterase and Russel viper-venom diesterase. These were purified preparations kindly given by Dr G. B. Petersen. For incubations of nucleotides with these enzymes the procedures of Burton & Petersen (1960) were followed.

Spectrophotometric measurements. For the range 350–850 μ a Unicam SP. 600 spectrophotometer was generally used. For some measurements in this range, and all measurements in the ultraviolet range, a Unicam SP. 500 or a Beckman model DU spectrophotometer was used. The extinctions of nucleotide solutions were measured against water in quartz cells of 1 cm. light path.

In calculating the quantities of nucleotides present in solutions, use was made of the molar-extinction coefficients quoted by Beaven, Holiday & Johnson (1955). To estimate the approximate amounts of nucleotides in crude extracts, the extinction at 260 μ was measured and a value of 10 used for the millimolar extinction coefficient.

Paper chromatography. This was carried out in all-glass tanks with Whatman no. 1 paper. When quantitative estimations of nucleotides were required the paper was washed in 2 N-acetic acid, followed by six changes of water.

Nucleotides were chromatographed in the following solvent systems; neutral or acidic (pH 3.8) ammonium acetate-ethanol (Paladini & Leloir, 1952); *iso*-butyric acid- NH_3 (Krebs & Hems, 1953); propan-1-ol- NH_3 soln. (sp.gr. 0.880)-water (6:3:1, by vol.; Hanes & Isherwood, 1949).

Sugars were chromatographed in butanol-ethanol-water (4:1:5, by vol.), phenol saturated with water or ethyl acetate-pyridine-water (2:1:2, by vol.).

Detection. Ultraviolet-absorbing compounds were located either by visual inspection under ultraviolet light or by making contact prints with unfiltered light from a mercury lamp (Markham & Smith, 1949). Reducing sugars were detected with the aniline hydrogen phthalate spray of Partridge (1949). N-Acetylaminosugars were located with

Table 1. *Nucleotide content of tissue extracts*

Abbreviations used: CMP, cytosine monophosphate; AMP, adenosine monophosphate; GMP, guanosine monophosphate; UMP, uridine monophosphate; ADP, adenosine diphosphate; ATP, uridine diphosphate; ATP, adenosine triphosphate. The exact identities of the compounds designated 'AMP' were not determined, but they were presumed to be isomers of AMP with possibly some adenine diphosphopyridine nucleotide.

Species	Tissue	Extraction method	Preliminary purification procedure*	Total nucleotides eluted (μ moles/g.)	Nucleotides eluted (μ moles/g.)								Unidentified
					CMP	'AMP'	GMP	UMP	ADP	UDP	UDPX	ATP	
<i>H. vulgaris</i>	Hepatopancreas	Perchloric acid	Ba pptn. IR-100	0.74	0.203†	0.011	0.011	0.059†	0.25	0.22	0	0	0
	Hepatopancreas	Perchloric acid	Charcoal	1.19	0.37†	0.04	0.04	0.07†	0	0.32	0.39	0	0
	Hepatopancreas	Perchloric acid	Charcoal Ba pptn. IR-100	0.4	0.19†	0.04	0.04	0.02†	0	0.15	0	0	0
	Hepatopancreas	Perchloric acid	Ba pptn. IR-100	0.93	0.17†	0.18	0.18	0.08†	0	0.50	0	0	0
	Hepatopancreas	Water then perchloric acid	Ba pptn. IR-100	6.98	1.04	1.0	2.38	1.31	0.15	0	0.75	0.22	0.13
Hepatopancreas		Water then perchloric acid	Ba pptn. IR-100	5.5	0.96	0.99	1.74	0.77	0.10	0	0.65	0.16	0.13
	Hypodermis	Perchloric acid	Ba pptn. IR-100	0.2	0.08	0.03	0	0	0.01	0	0.08	0	0
	Hypodermis	Perchloric acid	Ba pptn. K ₂ SO ₄	0.14	0.06	0.01	0	0	0	0	0.07	0	0
	Hypodermis	Water	Ba pptn. IR-100	1.13	0.39†	0.09	0	0	0.48	0	0.15	0.022	0
<i>C. macrurus</i>	Hypodermis	Perchloric acid	Ba pptn. IR-100	0.11	0.08†	0	0	0	0	0.03	0	0	0
	Hypodermis	Perchloric acid	Ba pptn. IR-100	0.02	0.01†	0	0	0	0	0.01	0	0	0
	Hypodermis	Water	Charcoal	1.75	0	0.19	0.18	0.58	0	0.27	0.08	0.45	0.45
	Hypodermis	Water	Phenol extraction Ba pptn. IR-100	2.22	0.71†	0.13	0.28	0.26	0.20	0.23	0.08	0.33	0.33

* For details see Methods.

† Complete resolution not attained.

the modified Elson and Morgan spray reagent (Partridge, 1948), and free (non-*N*-acetylated) amino sugars and amino acids with ninhydrin (0.4% in butan-1-ol saturated with water).

Paper electrophoresis. This was carried out at room temperature in a Perspex apparatus made by Shandon,

London. Whatman no. 1 paper (8 cm. × 32 cm.) held between two glass plates was used.

Reference nucleotides. Uridine diphosphate *N*-acetylglucosamine, uridine 3'-monophosphate and uridine 5'-monophosphate were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A.

Fig. 1. Dowex-1 fractionation of lobster-hepatopancreas extracts. Resin columns 15 cm. high × 0.5 cm. diam. with the acid formate-eluting system of Hurlbert *et al.* (1954) were used. (i) Total barium salts from 3.6 g. wet wt. of tissue extracted with cold perchloric acid. (ii) Total barium salts from 1.3 g. wet wt. of tissue extracted with boiling water.

RESULTS

Nucleotide content of tissue extracts

Considerable difficulty was experienced in obtaining reproducible column chromatograms of tissue extracts, especially those of *Carcinus* hypodermis. A comparison of various extraction and purification procedures was therefore carried out in order to determine the most favourable conditions for obtaining high yields of nucleotides.

Fractionation by column and paper chromatography of the hypodermis and hepatopancreas extracts revealed the presence of mixtures of nucleotides, including *N*-acetylhexosamine derivatives of uridine diphosphate. The results of a number of experiments are summarized in Table 1, and a comparison between two experiments is given in Fig. 1. In all cases where the methods were compared, extracts made with hot water contained a wider range and larger quantities of nucleotides per g. wet weight of tissue than those prepared by extraction with perchloric acid. (Expression of the nucleotide contents on a wet-weight basis was only satisfactory for hepatopancreas tissue, which can be easily weighed before extraction; lobster hypodermis always retained some fragments of muscle after the necessarily rapid dissection, and *Carcinus* hypodermis was difficult to remove without a quantity of fluid simultaneously draining out of the carapace. The recorded wet weights of hypodermal tissues are therefore high, but serve as a rough guide within the series of experiments with a particular species.) Purification of the nucleotides via their barium salts and removal of the barium either with Amberlite IR-100 or as BaSO₄ made little difference to the overall yields. With the heavily pigmented aqueous extracts of *Carcinus* hypodermis, purification of the nucleotides as their barium salts was inadequate for obtaining satisfactory fractionations on Dowex-1; in these cases it was found that preliminary treatment with phenol or charcoal was essential, although experiments with other tissue extracts indicated that appreciable losses of nucleotides may occur in such cases. The qualitative differences in nucleotide contents for the two extraction methods were reasonably consistent for

all the tissues. Perchloric acid extracts usually contained a high proportion of the amino sugar-containing nucleotides designated UDPX, with cytidine monophosphate and adenosine monophosphate as the other main constituents; only traces of uridine monophosphate and guanosine monophosphate, with some adenosine diphosphate, uridine diphosphate and adenosine triphosphate, were usually detected. By contrast, in aqueous extracts the proportion of UDPX was of the order 10–12%, with corresponding increases in the other constituents. Nucleoside triphosphates, with the exception of adenosine triphosphate, were not detected in significant quantity in any of the extracts examined. It was concluded that with these tissues extraction with hot water provides a very much more efficient means of obtaining nucleotides than does treatment with cold perchloric acid. The reasons for this difference were not determined.

Structural investigations on N-acetyl amino sugar nucleotide fractions

Homogeneity of compounds. After isolation by ion-exchange chromatography, the nucleotides were rechromatographed on Dowex-1 with eluting systems different from those used in the original separations; the homogeneity with respect to ultraviolet-absorbing material was then further checked by paper chromatography. The compounds corresponded to those designated UDPX in Table 1, and also included similar substances isolated from the hypodermis of *M. squinado* and the hepatopancreas of *C. maenas*. Since the same results were obtained with each of the nucleotides isolated from the five different tissues, it seemed likely that these compounds were identical; the structural investigations are therefore described in terms of a single substance.

In each experiment the compound possessed a typical uridine ultraviolet-absorption spectrum which was abolished by bromine in acid solution (Caputto, Leloir, Cardini & Paladini, 1950); hydrolysis with 2*N*-HCl at 100° for 6 hr. yielded uracil. Two molecules of phosphate were present per molecule of uridine (Table 2). Hydrolysis with 0.1*N*-HCl at 100° for 30 min. released uridine

Table 2. *Properties of UDPX nucleotides*

Source of nucleotide ...	<i>Carcinus maenas</i>		<i>Homarus vulgaris</i>		<i>Maia squinado</i>
	Hypodermis	Hepatopancreas	Hypodermis	Hepatopancreas	Hypodermis
Uridine	1.0	1.0	1.0	1.0	1.0
Total phosphate	1.98	2.08	1.96	1.89	1.74
'Labile phosphate'	0.99	—	—	1.19	—
<i>N</i> -Acetyl amino sugar					
Borate method	1.0	1.16	1.0	0.91	0.91
Carbonate method	0.84	1.23	0.98	0.62	—

5'-phosphate, which was identified by paper electrophoresis in 0.1M-borate buffer at 7v/cm. for 6-7 hr. (Crestfield & Allen, 1955), and by paper chromatography in neutral ammonium acetate-ethanol, with authentic uridine 5'-phosphate and uridine 3'-phosphate as markers. Hydrolysis with 0.01N-HCl at 100° for 5 min. released uridine diphosphate, which was identified chromatographically by comparison with the product obtained from uridine diphosphate *N*-acetylglucosamine under the same conditions of hydrolysis (Cabib *et al.* 1953).

A positive Morgan and Elson reaction was obtained only after hydrolysis with 0.01N- or 0.1N-HCl at 100°, the substance giving the positive test coinciding with the ultraviolet-absorbing areas on paper chromatograms. Calibrated for *N*-acetylglucosamine, the borate method indicated a 1:1 ratio for *N*-acetyl amino sugar/uridine, the spectrum of the chromogen closely resembling that given by *N*-acetylglucosamine (Table 2). There was no increase in this apparent ratio when the carbonate method of Aminoff *et al.* (1952) was used, and the chromogen's spectrum again resembled that given by *N*-acetylglucosamine. It was therefore concluded that stable 3-*O*-substituted derivatives of *N*-acetylglucosamine were absent (Cifonelli & Dorfman, 1957). Hydrolysis with 2N-HCl at 100° for 2 hr. produced a substance which moved with glucosamine and gave positive tests for amino sugars on paper chromatograms. Ninhydrin treatment of this amino sugar produced arabinose (Stoffyn & Jeanloz, 1954), indicating that the parent sugar was either glucosamine or mannosamine. To distinguish between these two possibilities, the products of hydrolysis of the nucleotides with 0.1N-HCl were chromatographed on borate-treated paper with butan-1-ol-pyridine-water (6:4:3, by vol.), which is reported to separate *N*-acetylglucosamine and *N*-acetylmannosamine (Comb & Roseman, 1958); the modified Elson and Morgan spray reagent revealed only a compound moving with *N*-acetylglucosamine. With a number of other solvent systems, only glucosamine or *N*-acetylglucosamine was detected on paper chromatograms of acidic hydrolysates of the nucleotide.

To test for amino acids, the compound was treated with 6N-HCl at 100° for 15-18 hr. and the hydrolysates were examined by paper chromatography (phenol saturated with water). Preparations of the nucleotide from lobster and *M. squinado* hypodermis were never obtained completely free from traces of amino acids, although none was detected in the purified substance isolated from the other three sources.

Hydroxamate tests for carboxylic esters in the intact nucleotide gave negative results.

Treatment of the compound with snake-venom diesterase followed by phosphomonoesterase released a substance which gave a positive Morgan and Elson test and which moved with *N*-acetylglucosamine on paper chromatograms.

Chromatographic behaviour of the nucleotide. In early experiments, certain preparations of the crustacean amino sugar-containing nucleotide, although closely resembling uridine diphosphate *N*-acetylglucosamine in its reactions, appeared to differ from it chromatographically (Kent & Lunt, 1958). However, it has subsequently been found that the nucleotide did not separate from uridine diphosphate *N*-acetylglucosamine when co-chromatographed with the latter on paper, or on chloride columns, although high salt concentrations (0.06N-NaCl in 0.01N-HCl) were required for elution. By contrast, during the preparation of a sample of uridine diphosphate *N*-acetylglucosamine from pig liver, it had been found, in agreement with Cabib *et al.* (1953), that uridine diphosphate *N*-acetylglucosamine required only 0.03N-NaCl in 0.01N-HCl for displacement from Dowex-1 chloride columns.

It was concluded that the actual chloride concentration required for elution did not necessarily provide a reliable means of identification of nucleotides on ion-exchange chromatography, although the order of appearance of compounds in column eluates proved to be reproducible under the conditions described above.

From these results it was concluded that each crustacean amino sugar-containing nucleotide was probably uridine diphosphate *N*-acetylglucosamine.

DISCUSSION

The most frequently investigated form of chitin is that of crab and lobster, where with proteins it forms the basis of the organic matrix of the exoskeleton. Biosynthesis of this tissue is believed to occur in the hypodermis, a thin cellular layer underlying the cuticle itself. However, comparatively little information is available about the biochemical activities of the hypodermis, the study of which is complicated by its high content of phenolic substances. There are indications in this tissue of *N*-acetyl- β -glucosaminidase and chitinase (Kuhn & Tiedman, 1954; Lunt & Kent, 1960), but although these enzymes may be significant in the reabsorption of chitin, it appears unlikely that they possess any significant biosynthetic function (Strominger, 1960).

The present studies show that the hypodermis contains a number of ribose nucleotides, including the monophosphates of cytidine, uridine, guanosine and adenine, and the diphosphates of adenine and uridine, as well as adenosine triphosphate. In

the hypodermis of each of the species investigated there is, however, only one amino sugar-containing nucleotide, as judged by ion-exchange and paper-chromatographic procedures. Degradation of this product yielded only uridine monophosphate, phosphoric acid and *N*-acetylglucosamine and it is considered to be uridine diphosphate *N*-acetylglucosamine or a closely allied substance. It therefore seems probable that the formation of the chitin of the exoskeleton occurs through the intermediary of this nucleotide, as with *Neurospora crassa* (Glaser & Brown, 1957). This nucleotide is present in appreciable quantity in the hepatopancreas of the lobster (0.15–0.75 μ mole/g. wet wt. of tissue) and of *C. maenas* (0.22 μ mole/g. wet wt. of tissue) and has been isolated from the hypodermis of the crab *M. squinado*.

The quantitative and qualitative yields of nucleotides varied according to the extraction procedures used, hot water being superior to cold perchloric acid in these experiments. With heavily pigmented tissues, substances which interfere with chromatographic procedures could be removed by phenolic extraction or by adsorption of the nucleotides on to charcoal, followed by elution with ethanolic ammonia solutions.

SUMMARY

1. Methods for the extraction and chromatographic fractionation of ribonucleoside phosphates from crustacean tissues are described.

2. Evidence is presented for the occurrence in these tissues of a uridine diphosphate amino sugar nucleotide, which is probably identical with uridine diphosphate *N*-acetylglucosamine.

3. The nucleotide is present in appreciable quantities in the hypodermis and hepatopancreas of *Carcinus maenas* and *Homarus vulgaris*, and in the hypodermis of *Maia squinado*.

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