cluding citrate, α -oxoglutarate, succinate, fumarate and malate, only glucose, fructose, lactate and pyruvate produced maximal mitotic activity over 4 hr. in mouse-ear skin *in vitro*.

The effects of glucose 1-phosphate are difficult to interpret without further experiments to determine the fate of the compound.

Barron *et al.* (1948) and Griesemer & Gould (1954) found citrate to have no effect upon skin respiration. Barron *et al.* also failed to demonstrate isocitric dehydrogenase in skin and found no stimulation on the addition of α -oxoglutarate. On these grounds they suggested that the citric acid cycle in its classical form did not operate in skin. Our results, which show a sharp contrast between the obvious usage of succinate, fumarate, malate and oxaloacetate and the transient usage of citrate and non-utilization of α -oxoglutarate, are consistent with their findings, but the evidence is as yet insufficient to conclude that skin differs in its oxidative processes from the majority of other tissues.

SUMMARY

1. The respiration and glucose utilization of skin of the guinea-pig ear *in vitro* are unaffected by the glucose concentration within the range 0.005–0.02 M.

2. The respiration and fructose utilization increase as the fructose concentration is increased.

3. In glucose–fructose mixtures the utilization of fructose is reduced, the glucose utilization is unaffected and the total hexose utilization thus increases. This is not reflected in an increased respiration rate.

4. In the absence of serum the glucose utilization is only about one-half of that occurring in the presence of serum, and the respiration is only slightly reduced; the sum of lactic acid produced and the calculated glucose oxidized equals the observed glucose utilized.

5. Glucose, fructose, lactate and pyruvate stimulate respiration and maintain a relatively high respiration rate for 24 hr.

6. Succinate, malate and acetate stimulate respiration but permit a gradual decline over 24 hr.

7. Oxaloacetate maintains respiration for 5 hr. only, subsequently becoming toxic; fumarate also stimulates respiration initially but its effect declines between 5 and 24 hr.

8. Citrate causes transitory stimulation in the first hour and α -oxoglutarate is without effect.

9. Glucose 1-phosphate causes initial depression, but at 24 hr. a slight increase over the control occurs.

10. These findings are discussed in relation to the carbohydrate metabolism of skin.

We are indebted to Professor J. R. Squire for his continued interest and advice.

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The Origin of Melezitose: a Biochemical Relationship between the Lime Tree (*Tilia* spp.) and an Aphis (*Eucallipterus tiliae* L.)

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The trisaccharide melezitose [O - α -D-glucopyranosyl-(1 $\rightarrow$ 3)- β -D-fructofuranosyl-(2 $\rightarrow$ 1) α -D-glucopyranoside] was first recognized as a new sugar by Bonastre (1833), who found it in a crystalline deposit called 'manna of Briançon', occurring on the

branches of the larch (*Larix* spp.; French, 'le mélèze'). It was later found in a number of other mannas and honeydews (listed by Hudson, 1946), notably that from Tashkent and the Middle East, called 'turanjabin'. Using material from the latter source Berthelot (1859), Alekhine (1889) and Tanret (1906) elucidated the main features of its

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structure. However, it was not until Hudson (1946) had pointed to a gap in the evidence that conclusive proof was obtained by Hehre for the substituted sucrose structure (Hehre & Carlson, 1952).

In 1917 melezitose was first noticed in honey. Hudson & Sherwood (1920) found that it was responsible for the crystallization of certain honeys in the comb (with disastrous results for the bees), and Dr E. T. Wherry (see Hudson & Sherwood, 1920) obtained evidence which indicated that the bees had obtained the melezitose by collecting honeydew from pine trees (*Pinus virginiana* Mill.) in the locality. These trees were infested with aphids and scale insects and the excretion of *Lachnus pineti* (Fab.) Koch appeared to be the main source of the trisaccharide. Later melezitose was found in European honeys, as the result of the collection of honeydew from conifers and lime trees (Nottbohm & Lucius, 1929, 1931) and from spruce (Gorbach, 1937). The absence of melezitose from nectar honeys suggested that it was not formed in the elaboration of nectar by the plant, nor in the processing and ripening of the honey by the bees. Thus although 'melezitose honey' is the immediate commercial source of the sugar (cf. Hudson, 1946), its origin is to be sought in the honeydews from which this honey is made.

There has been a divergence of opinion about the origin of honeydews and mannas, which may be defined respectively as the fluid and solid deposits, rich in sugar, found on the leaves and branches of various trees and shrubs. Some writers regarded them as the result of normal physiological processes in the plant under special climatic conditions (e.g. Davidson, 1919), while others (e.g. Alekhine, 1889; Maquenne, 1893), although alive to the possibility that insects were responsible, could not find any on the plant material they examined. The prevailing view is, however, that insects are always responsible (cf. Tanret, 1920); they puncture the plant tissues and allow the sap to flow into their alimentary tract. They absorb some of the sap constituents and expel the unwanted fluid from the anus from time to time, usually in such a manner that it falls away from the place at which they are feeding (see, for example, Broadbent, 1951).

If it is admitted that honeydews and mannas are always produced by insects, the sugars found in them may still be conceived to originate in a number of ways: for example, (a) they could be present in the normal sap of the plant; (b) they could be produced by plant tissue damaged or otherwise modified by the proboscis of the insect; (c) they could be produced by the insect from the sugars present in the normal sap. Hudson (1946) was inclined to believe that melezitose was already present in the sap of the various species of plant concerned, but other workers (e.g. Gorbach; letter to Mr J. Pryce-Jones, 1950)

came to the conclusion that the insect must be responsible, since no melezitose could be found in the sap of trees (spruce, oak) on which melezitose-containing honeydew was formed.

In 1952 Dr T. E. Mittler (cf. Mittler, 1954) found that a trisaccharide was present in the honeydew of an aphid, *Tuberolachnus salignus* Gmelin, feeding on willow (*Salix* spp.), but not in the sap collected from the cut ends of the insect's stylets by the technique of Kennedy & Mittler (1953). The trisaccharide had an R_f similar to that of melezitose, and gave glucose and fructose on hydrolysis. These properties in themselves are not sufficient to identify the substance as melezitose (3^F - α -glucosylsucrose), because in 1953 a trisaccharide with similar properties, 4^G - α -glucosylsucrose ('maltosylfructoside') was found to be produced by honey invertase (White & Maher, 1953), and to be present in the excreta of various insects, including the honeydew of *Aphis spiraeicola* (Patch) (Gray & Fraenkel, 1953, 1954). Duspiva (1953, 1954) studied the action of extracts of various parts of the gut of aphids on sucrose, and suggested that the mixture of sugars found in honeydew represented an intermediate stage in the action of digestive enzymes of the insect, which included transglycosylation. However, he did not produce any specific evidence that melezitose was formed in this way.

After preliminary experiments in 1954 with enzyme preparations from aphids, in which 4^G - α -glucosylsucrose, but no melezitose, was produced from sucrose, and an examination of the sugars of the leaves of larch and other trees, we decided to examine more closely the production of melezitose-containing honeydew by aphids feeding on the lime tree (*Tilia*). Lime-tree honeydew contains relatively large amounts of melezitose (Maquenne, 1893), and is produced in abundance in this country under the appropriate but infrequent weather conditions, namely a succession of hot rainless days.

We have found that there is little, if any, melezitose in the leaves of the tree. On the other hand, the bodies of the aphids which produce the honeydew contain an enzyme which will form melezitose from sucrose. A preliminary account of this work has been given to the Biochemical Society (Bacon & Dickinson, 1955).

After an investigation of honeydew production on citrus plants Wolf & Ewart (1955*b*) have briefly reported that the melezitose-containing honeydew of a coccid (*Icerya purchasi* Mask.) contains also an enzyme capable of forming melezitose from sucrose.

METHODS

The methods of analysis used, including the chromatography of sugars, were essentially those already described (Bacon, 1954). The use of a diphenylamine-aniline-phosphoric acid dip (cf. Harris & MacWilliam, 1954), which was stated by

Saroja, Venkataraman & Giri (1955) to distinguish 1:4- from 1:6-linked glucose oligosaccharides, was found to distinguish very satisfactorily between melezitose and 4^α-α-glucosylsucrose on paper chromatograms. When heated at about 95–100° for 1–2 min. melezitose gave at first a grey colour, which quickly changed to a brown, that persisted on storage away from light; 4^α-α-glucosylsucrose gave a greenish blue colour that was also stable for long periods if kept in the dark. The colours given by raffinose and 1^β-β-fructosylsucrose, although not easily described, could be distinguished from each other and those given by the other two trisaccharides.

MATERIALS

Melezitose. Commercial samples (Edward Gurr Ltd., London) were used without purification for most purposes. A sample of the trisaccharide kindly sent by Dr Hewitt G. Fletcher, jun., of the National Institutes of Health, Bethesda, Maryland, U.S.A., was used as reference material in the characterization of enzymically synthesized melezitose.

4^α-α-Glucosylsucrose. A small amount of a syrup having the properties of this compound was isolated from an incubation of crushed greenflies with sucrose solution: 115 mg. of whole unidentified green aphids was crushed in 1 ml. of water, 4 ml. of water containing 2 g. of sucrose was added and the mixture (pH about 6.5) left (with chloroform) at room temperature for 2 days. The reaction was terminated by heating at 100° for 10 min. and the mixture fractionated on an Activated Charcoal (B.D.H. Ltd.)–Celite column (40 g.:40 g.) by gradient elution [50% (w/v) ethanol into 1 l. of water]. The first 500 ml. of effluent was discarded; the next 85 ml. contained the trisaccharide and was evaporated to dryness. The material (a syrup) was non-reducing, and on hydrolysis with 0.5% oxalic acid at 100° for 30 min., or with yeast invertase, it yielded fructose and a disaccharide having the same *R_F* as maltose and giving the characteristic blue colour given by maltose with the diphenylamine–aniline–phosphoric acid reagent. It was further characterized by the tests applied to a similar substance isolated from incubations with *Eucallipterus tiliæ* (see below).

Turanose. A small sample was obtained from Dr N. K. Richtmyer, part of a gift from him to Dr D. J. Bell.

Leaves. Leaves were collected from various lime trees, including some in an avenue at Tissington, Derbyshire, and others in the Botanical Gardens, Sheffield. The species of lime commonly found in England are *Tilia platyphylla* Scop., *T. cordata* Mill. and a hybrid between them, *T. × vulgaris* Hayne. Most of the leaves collected were referable to *T. platyphylla* or *T. vulgaris*, but some did not fall easily into this classification.

The leaves were cooled soon after collection. Twigs bearing leaves were cut from trees growing near the laboratory and transferred as quickly as possible to the deep-freeze. Once frozen the petioles became so brittle that the leaves could be snapped off. Leaves which had to be brought from greater distances were frozen with solid CO₂ in a large vacuum flask and later transferred to the deep-freeze.

In this way the leaves of *Tilia* (and of other trees) could be collected during hot sunny periods, when the carbohydrate content should be at its highest, and if necessary preserved for a few weeks before being analysed.

Germination of larch seeds. Seeds of the larch, *Larix leptolepis* (Sieb. & Zucc.) Gord. were spread on a pad of

filter paper, kept moist by capillarity through a 'wick' of filter-paper strips, and covered with an inverted glass funnel. After 4 weeks at 30°, the sprouts were up to 8 cm. long and yellow at the tops, but the root systems were not very well developed.

Aphids. *Eucallipterus tiliæ*, a yellow to orange, spotted aphid, is usually found on the undersurfaces of the leaves although a few individuals crouch in the depressions formed by the ribs on the upper sides. While the trees are in blossom a proportion of the aphids attach themselves to the flower stalks. The number on each leaf is always small, so that a large number of leaves have to be explored in order to collect an appreciable weight of insects. When touched with a pointed instrument the insect will often spring into the air and fall into a suitable receptacle, but equally often moves inexpertly sideways and becomes stuck to the leaf by its own excreta. Once transferred to a glass vessel on a warm afternoon it becomes very active. The various difficulties were overcome by placing a 500 ml. beaker inside a larger vessel and packing cotton wool into the space between them. A few drops of chloroform were placed on the cotton wool from time to time, and this served to kill or at least immobilize most of the insects which fell into the beaker. Inevitably other insects were included, and small pieces of vegetation, blossoms, etc. At the end of a period of collection the entire contents were spread on a sheet of paper and the unwanted material was removed. The specimens of *E. tiliæ*, which included many wingless and immature forms, were then transferred to a glass-stoppered tube, weighed, and stored in the deep-freeze; they retained their enzymic activity for at least one year. After 2–3 hr. spent in collecting, transporting and sorting the material the yield was usually of the order of 100 mg. fresh wt. of whole insects.

Honeydew. Since most of the aphids live underneath the leaves the bulk of their excretion falls on to the objects immediately below them, chiefly the upper surfaces of other leaves. Honeydew was collected by rubbing the upper surface of each leaf several times with a piece of cotton wool soaked with water. The cotton wool was frequently washed in 50–100 ml. of water held in a beaker. The washings were transferred to a bottle and a few drops of chloroform at once added. In Sheffield the washings so obtained were turbid, and contained black particles. This contamination would appear to be a result of air pollution by industrial processes, and not of mould growth. Most of the colour and turbidity was removed by filtration through Whatman no. 1 paper. The pH was usually low (about 4).

In a single experiment a sheet of transparent material (Pliofilm) was spread across a lower branch of a lime tree and left for 5 hr. (12.00 to 17.00 hr.). It was then carefully folded and transported to the laboratory, where the honeydew was washed from the upper surface. On evaporation the washings yielded 140 mg. of residue from 1.7 m.².

By the use of small pieces of Pliofilm attached to individual leaves we were able to satisfy ourselves (a) that honeydew fell on to the upper surfaces of leaves, and was not secreted by them, and (b) that *E. tiliæ* was chiefly, perhaps entirely, responsible for the honeydew, relatively large droplets of honeydew appearing on the film below each individual within a few hours.

Other insects and honeydews. The collection of small samples of other insects and their excreta is referred to in the next section.

RESULTS

Sugars in extracts of leaves

Leaves were extracted by dropping them into boiling water and continuing the boiling for 5–10 min. after the last addition. The pH of such an extract was tested in a preliminary experiment, and was usually greater than 5. If the pH was raised by the addition of alkali or buffer subsequent operations were complicated by the extraction of gelatinous, presumably pectic, material; such additions were therefore avoided unless the pH of an extract fell below 5. Paper chromatograms of these aqueous extracts ran badly, and Activated Charcoal–Celite columns became blocked, unless a preliminary precipitation with about 50 % ethanol was carried out. With lime leaves the ethanol precipitate was mucilaginous, but with the other leaves examined it had the properties of a pectic acid gel. The following is a typical procedure.

Leaves of *T. vulgaris* (50 g.), collected 11 July 1955, were dropped into 200 ml. of boiling water. A few drops of 5 % (w/v) Na_2CO_3 were added to keep the colour reaction blue to bromocresol green, and about 50 ml. of water to replace that lost by evaporation. Boiling was continued for 5 min. after the last addition, the whole cooled and transferred to a Waring Blender. After blending for a few minutes the extract was separated from the leaf debris by squeezing through heavy cotton cloth, and an equal volume of ethanol added to the filtrate. After the precipitate had flocculated it was removed by filtration through a Green's no. 904½ pleated filter paper, and the filtrate evaporated to dryness *in vacuo* with a bath temperature not exceeding 50°. The residue was then dissolved in a small volume of water for chromatographic analysis.

Lime. Paper chromatography showed the presence of much sucrose, smaller amounts of glucose, and relatively little fructose. There were traces of other disaccharides, and material in the positions of melezitose and raffinose. The extracts were fractionated by gradient elution on Activated Charcoal–Celite columns, with or without a preliminary removal of monosaccharide on a similar column (Bacon, 1954). From 40 g. of leaves was obtained in one such separation about 1 g. of crystalline sucrose, but the yields of the other oligosaccharides were much lower. All the fractions following sucrose were therefore usually bulked and fractionated by paper chromatography in *n*-butanol–acetic acid–water. The various components were extracted as described by Allen & Bacon (1956). Treatment with 0.5 % oxalic acid at 100°, or with yeast invertase at room temperature, combined with paper chromatography, was employed in an attempt to detect melezitose among these components. When dissolved in water equivalent to

0.02 times the original fresh weight of leaf the non-sucrose oligosaccharide fraction always showed a trisaccharide with the R_f of melezitose, in amounts corresponding to about 0.5 % of the solution. However, when this substance was separated and treated with yeast invertase appreciable amounts of fructose and glucose were produced, leaving invertase-resistant trisaccharide amounting to only about one-fifth of the original. The material having the properties of melezitose thus amounted to only about 1 mg. in 50 g. of leaves, as compared with 1 g. of sucrose.

By similar techniques raffinose was identified in the extracts. There were also present traces of other oligosaccharides, including one giving a blue colour with the diphenylamine–aniline–phosphoric acid reagent but having an R_f rather smaller than that of maltose, in butanol–acetic acid–water.

Larch. Extracts of young leaves, collected 9 May 1955, contained sucrose and glucose and small amounts of trisaccharide. The oligosaccharide fraction (Active Carbon no. 130, Sutcliffe, Speakman and Co. Ltd., Leigh, Lancs.) from 10 g. of leaves weighed 70–80 mg. The trisaccharide was seen as a single spot with the R_f of raffinose when the fraction was dissolved in 0.5 ml. of water and chromatographed. There was no sign of material with the R_f of melezitose, and after oxalic acid hydrolysis no ketose-reacting material remained in the disaccharide position (see below).

Twenty seedlings (660 mg.) of *Larix leptolepis*, were extracted with boiling 50 % ethanol and the extract was examined by paper chromatography before and after the separation of the oligosaccharide fraction on an Active Carbon no. 130–Celite column. No substance with the R_f value of melezitose was detected.

Ash. Sucrose, glucose and fructose were found, with appreciable amounts of higher oligosaccharides. These appeared to be raffinose, stachyose and verbascose (cf. French & Wild, 1953), the first two having the same R_f values as authentic raffinose and stachyose (Bealing, 1953), and all three containing ketose, presumably fructose, removable by hydrolysis with oxalic acid or by yeast invertase. Stachyose predominated; there was no sign of a member of the series higher than verbascose. There was also no sign of melezitose.

Willow. A spot of willow sap collected (24 May 1954) by Mr G. P. Hill, by the method of Kennedy & Mittler (1953), showed only sucrose, confirming the observation of Mittler (1954).

Sugars of honeydews

Lime. Honeydew was washed off lime leaves collected in June 1949 and the washings were filtered through a little charcoal, concentrated and treated with an equal volume of ethanol. The

alcoholic supernatant on evaporation *in vacuo* yielded a syrup. A sample of this (about 1 g.) was fractionated by gradient elution on Activated Charcoal–Celite. Crude samples of sucrose (202 mg.) and melezitose (304 mg.) were obtained, from which 115 mg. and 244 mg. respectively of crystalline material were obtained (sucrose $[\alpha]_D + 65.6^\circ$; melezitose $[\alpha]_D + 90.2^\circ$).

The fractionation also gave about 40 mg. of a mixed trisaccharide fraction in which raffinose and 4^G- α -glucosylsucrose were tentatively identified.

These results were confirmed and extended by an analysis of honeydew collected 14 July 1955. The washings from the lower leaves of a tree of *Tilia vulgaris* were filtered and evaporated to small bulk *in vacuo* after adjustment of the pH to about 6.5. The concentrate was applied to an Activated Charcoal–Celite column (40 g.: 40 g.) and developed by gradient elution, ethanol (50 %, v/v) dropping into 1 l. of water. This did not achieve a complete separation of the sugars. The mixed fractions, each of which consisted of two main components, were therefore analysed as follows. The monosaccharide was estimated as reducing sugar (Miller & Van Slyke, 1936) and its fructose component by Cole's method (Bacon & Bell, 1948). Quantitative paper chromatography (Bealing & Bacon, 1953) was applied directly to the mixture of sucrose and melezitose, and to the mixture of melezitose and presumed 4^G- α -glucosylsucrose after it had been treated with yeast invertase, which yielded free fructose and a resistant trisaccharide. The results are given in Table 1. In addition to the substances listed a small amount of a disaccharide having the chromatographic behaviour of maltose was eluted with the mixed sucrose–melezitose fractions. Evidence was also obtained (cf. the preceding section) for the presence of small amounts of raffinose in the 4^G- α -glucosylsucrose fractions.

The 4^G- α -glucosylsucrose was characterized only by paper chromatography, hydrolysis with yeast invertase giving a monosaccharide with the chro-

matographic behaviour of fructose, and a disaccharide which resembled maltose.

Lime-tree honeydew from scale insects. In May 1956 honeydew was noticed on a lime tree which appeared to be almost free from *E. tiliae*. This honeydew was traced to a small number of scale insects (*Eulecanium coryli* L.), which feed on the twigs rather than the leaves of the tree. When examined by paper chromatography, before and after mild acid hydrolysis, it was found to be quite different from the *E. tiliae* honeydew. Like the latter it contained appreciable amounts of sucrose, glucose and fructose, but no melezitose could be detected, and in its place there were present a series of sugars which appeared, like those described by Wolf & Ewart (1955*a*), to belong to two series (their 'O' and 'M' series), namely that based on sucrose by the successive addition of α -1:4-glucosyl radicals to the glucose moiety, and the corresponding substances with fructose removed, i.e. the dextrin series. 4^G- α -Glucosylsucrose and maltose were present in relatively small amounts in the two samples of honeydew examined, compared with the upper members, of which at least four could be counted on chromatograms developed for long periods.

Ash (*Fraxinus* spp.). The excretion of a psyllid (*Psyllopsis fraxinicola* Frstr.) living on this tree is in the form of a thick syrup covered by an envelope (probably of a waxy nature). The honeydew thus consists of small pellets, which may dry and harden in the sun. A single pellet smeared on the starting line of a paper chromatogram provided ample material for the identification of its constituent sugars. These always included the three members of the raffinose series already identified in the leaf sap, but variable, sometimes almost insignificant, amounts of sucrose. Apart from traces of glucose and fructose, and possibly of melibiose, no other sugars were detectable with the reagents used.

Other trees. Washings from the leaves of a sycamore (*Acer pseudoplatanus* L.) showed the presence

Table 1. *Sugars of lime-leaf honeydew*

Chromatographic separation and subsequent analysis of mixed fractions is described in the text. The amount of each sugar (except glucose) is calculated from the result of a ketose estimation, and is given to the nearest milligram. Volumes of fractions 1–16 and 26–29, which contained no detectable carbohydrate, were 260 and 44 ml. respectively.

Fraction nos.	Volume (ml.)	Dry wt. (mg.)	Fructose	Glucose	Sucrose	Melezitose	4 ^G - α -Glucosylsucrose
17–25	100	180	113	34	—	—	—
30–38	85	370	—	—	308	—	—
39–42*	33	65	—	—	10	43	—
43–51	71	280	—	—	—	281	—
52–55	25	14	—	—	—	4	4
56–67†	93	31	—	—	—	—	21†
Totals (mg.)			113	34	318	328	25
Totals (as % sugar recovered)			14	4	39	40	3

* This fraction contained a small proportion of maltose.

† Contained some raffinose.

of a multiplicity of sugars; paper chromatograms of the unfractionated material were too complex to permit identification of anything but sucrose, glucose and fructose. There did not appear to be much, if any, melezitose, and apparently not much 4^α-α-glucosylsucrose. It is possible that some mould growth had taken place. The material was not examined further. *Drepanosiphum platanoides* Schr. was the most abundant insect on these leaves.

Action of extracts of insects on sucrose

Experiments of a preliminary nature were made with unidentified aphids taken from various garden plants. The insects were crushed with a glass rod in the presence of a small amount of sucrose solution (20–40%, w/v) and a few drops of chloroform, and the suspension, after addition of further sucrose solution, was left at room temperature (about 20°). The pH was sometimes controlled by the use of buffers, but otherwise was on the acid side of neutrality. The tougher parts, wings, legs, integument, etc., were not removed from the suspensions before incubation, but were separated incidentally to sampling and chromatography. From such incubation mixtures, sucrose disappeared at rates of the order of 1 mg./mg. fresh wt. of insect/day, and there accumulated a large number of oligosaccharides, which appeared to include 4^α-α-glucosylsucrose and maltose. A fractionation of one such incubation mixture, which provided a sample of 4^α-α-glucosylsucrose (see Materials), showed no sign of the presence of melezitose, nor could any be detected by paper chromatography.

Eucallipterus tiliæ L. In July and August 1955 similar experiments were carried out with *E. tiliæ*. Since the R_f values of melezitose and 4^α-α-glucosylsucrose in butanol-acetic acid-water are very similar, melezitose was tested for by heating a sample of the incubation mixture at 100° with 0.5% oxalic acid for 30 min., and spraying a paper chromatogram of the hydrolysate with phloroglucinol (Horrocks & Manning, 1949). Of the substances known or suspected to be present only melezitose yields a ketose-containing disaccharide (turano); the persistence of ketose in the disaccharide position was thus taken as evidence for the formation of melezitose. This was confirmed by developing chromatograms of the untreated incubation mixture for 3–4 days, and dipping them in the diphenylamine-aniline-phosphoric acid reagent. Although melezitose and 4^α-α-glucosylsucrose are not usually completely separated by this length of run, the reagent shows very clearly the difference in colour between the front and back of the spot. The front portion (melezitose) is brown and the back portion (4^α-α-glucosylsucrose) a dull blue.

These tests showed that sucrose incubated with crushed *E. tiliæ* gave fructose, glucose and a tri-

saccharide fraction which appeared to contain both melezitose and 4^α-α-glucosylsucrose. This supposition was confirmed by experiments on a larger scale.

Sucrose (3 g.) was dissolved in 12 ml. of water and 280 mg. of freshly collected whole insects was crushed in 0.5 ml. of the solution. A further 10 ml. of solution was added, and 0.5 ml. of the suspension withdrawn. The pH of the latter was adjusted to pH 5.0 with sodium acetate-acetic acid buffer, and 0.1 ml. of M-sodium potassium phosphate buffer (pH 6) was added to the larger portion. Chloroform was added to both, and samples (about 3 μl.) were withdrawn at intervals. After 67 hr. at room temperature 0.5 ml. was withdrawn from the pH 6 incubation mixture and the remainder inactivated by heating at 100° for 10 min.

Examination of the smaller incubation mixtures was continued for 11 days and showed that the action on sucrose was more rapid at pH 6 than at pH 5. As the reaction progressed appreciable amounts of substances reacting blue to the diphenylamine-aniline-phosphoric acid reagent were formed at both pH values. Melezitose and 4^α-α-glucosylsucrose were present in both incubations and there was no indication that their relative proportions varied with pH or the time of incubation.

The inactivated sample (9.5 ml.) was subjected to gradient elution on Activated Charcoal-Celite (42 g.:42 g.), 50% (v/v) ethanol dropping into 1 l. of water. The first 550 ml. of effluent was discarded and the fractions containing melezitose (the next 74 ml.) were taken to dryness and fractionated again on a smaller column (15 g.:15 g.), 50% (v/v) ethanol dropping into 500 ml. This yielded 97.8 mg. of a syrup which was treated with charcoal (recovery: 93.5 mg., $[\alpha]_D^{20} + 80^\circ$), dissolved in 0.5 ml. of 50% ethanol, warmed and 1.25 ml. of ethanol added. The substance crystallized when seeds of melezitose were added. The crystals (66 mg.) were dried in a desiccator over $\text{CaCl}_2\text{-NaOH}$: $[\alpha]_D^{20} + 88.5^\circ$. This material was recrystallized from 85% (v/v) aqueous ethanol for physical examination. It was identical both by infrared spectrum (Dr E. J. Bourne) and X-ray diffraction picture (Mr O. G. Griffin; see Addendum) with authentic melezitose monohydrate.

The fractions following melezitose in the first fractionation (198 ml.) contained 115 mg. of material, which appeared to be chiefly 4^α-α-glucosylsucrose, apart from traces of mono- and di-saccharide. The trisaccharide component was hydrolysed by yeast invertase to a mixture of fructose and maltose, and by a preparation of intestinal maltase freed from invertase activity by heat treatment (Bacon & Bacon, in preparation) to a mixture of sucrose and glucose; in both cases the products were identified only by their chromato-

graphic behaviour and reactions with spraying reagents. Gray & Fraenkel (1954) stated that their 'fructomaltose' (probably 4^g- α -glucosylsucrose) was hydrolysed by the butanol-acetic acid-water mixture of Partridge (1948). It seems possible that traces of mineral acid in their acetic acid were responsible. On one occasion in this laboratory a batch of this solvent made with laboratory reagent-grade acetic acid caused hydrolysis of sucrose and other fructosides. Since that time we have always used A.R. grade and have never noticed any hydrolysis of fructosides, including 4^g- α -glucosylsucrose, and it is very unlikely that the substance has, for this reason, escaped our notice in the *Psyllopsis fraxinicola* honeydew. Gray & Fraenkel also reported hydrolytic action by a pancreatic extract. We have found a slow hydrolysis by saliva. Probably in both cases maltase was responsible.

At this stage of the reaction no sugars other than fructose, glucose, sucrose and the two trisaccharides were present in sufficient amounts to merit attention, but higher oligosaccharides were detectable on chromatograms from more lengthy incubations.

DISCUSSION

It is interesting to compare the carbohydrate composition of honeydews of the psyllid (*Psyllopsis fraxinicola*), the aphid (*Eucallipterus tiliae*) and the scale insect (*Eulecanium coryli*), with the carbohydrates of the sap on which they feed. The psyllid utilizes the greater part of the sucrose and monosaccharide present in its food, but fails to attack the galactosylsucroses, raffinose, stachyose and verbas-cose. The aphid and scale insect, on the other hand, both excrete sucrose and monosaccharide, and with them appreciable amounts of sugars which are not demonstrable in the leaves on which they feed. Thus although all three excrete sugars, in the psyllid this would appear to be due to its lack of any enzymic mechanism (e.g. α -galactosidase or β -fructofuranosidase) by which raffinose and related compounds can be hydrolysed, whereas in the aphid and scale insect it is due to a failure to utilize fully the sucrose which is available.

One possible explanation of the presence of a high proportion of melezitose, 4^g- α -glucosylsucrose or related substances in honeydew is that they are present in the sap in much smaller proportions, but become concentrated relative to the other sugars by the preferential utilization of the latter (sucrose, monosaccharides, etc.). If one assumes that the small melezitose-like fraction of lime leaves described above is in fact melezitose, then to reduce the ratio of sucrose to melezitose, which is approximately 1000:1 in the leaf extract, to its value of about 1:1 in the honeydew, the insect would have to utilize 99.9 % of the sucrose.

This is hardly in keeping with the vast amounts of sugar excreted by aphids (cf. Mawson, 1948), which early led to the belief that excessive amounts of carbohydrate were inhibited in order to obtain sufficient nitrogen (a belief needing revision in the light of the discovery by Maltais & Auclair (1952) that aphids excrete also substantial amounts of amino acids).

The only quantitative study of the utilization of nitrogen and carbohydrate is that by Mittler (1954) using *Tuberolachnus salignus*. Kennedy & Mittler (1953) had found that phloem sap exudes from severed stylets at a rate very similar to that at which honeydew is excreted by adjacent aphids. Mittler (1953) measured the total nitrogen content of freshly excreted honeydew and found it always to be less than that of sap collected simultaneously. The difference was observed over a wide range of concentrations, depending upon the developmental stage of the plant (*Salix*), and, with other evidence, suggested that the insect absorbs some nitrogenous constituents of the sap, among them amino acids. However, he found that the concentration of total sugar in the honeydew did not differ greatly from that in the sap, the difference seldom exceeding 3 %. Absorption of carbohydrate, although not excluded by this observation, must be proportionately less than that of nitrogen, and would not be expected to lead by itself to any great changes in the relative proportions of the sugars present in the sap. It may also be noted that if raffinose is not attacked in the digestive system of the insect the relative amount of this trisaccharide would also be expected to increase. In fact, the chromatographic analysis showed only trace amounts of raffinose in the honeydew, perhaps 0.1 % of the total sugar.

A more convincing argument against this explanation is the striking difference between the carbohydrates excreted by the aphid and scale insect feeding on the same tree. In the honeydew of *Eulecanium coryli* no melezitose is detectable, but in its place there occur considerable amounts of sugars which are not seen at all in the leaf extracts. The conclusion seems inescapable that melezitose and the 4^g- α -glucosylsucrose family of substances are produced by the action of insects, and that the carbohydrates of their honeydews represent, as Duspiva (1953) first suggested, a stage of incomplete digestion of sucrose, a consequence of the superfluity of carbohydrate.

The possibility that the plant produces abnormal sugars when damaged by the mouthparts of the insect is difficult to exclude. However, like Mittler, we could find no trisaccharide in the sap collected from a severed stylet. The present experiments show that somewhere the insects carry an enzyme system capable of forming melezitose and 4^g- α -glucosylsucrose from sucrose. Wolf & Ewart (1955b) have shown that a scale insect (*Coccus*

hesperidum L.), which excretes 4^g- α -glucosylsucrose, excretes with it an enzyme capable of forming it from sucrose, whereas a species (*Icerya purchasi*) which excretes melezitose excretes a melezitose-forming enzyme. In these cases the enzymes responsible would thus seem to be present in the lumen of the alimentary tract, and hence it could be argued (but without much conviction) that they were selectively imbibed with the sap.

Although there is a little 4^g- α -glucosylsucrose in *Eucallipterus tiliae* honeydew, the major trisaccharide component is melezitose. It is not easy to reconcile this with the 1:1 ratio found in the *in vitro* incubations. It is possible that two enzymes are concerned and that they normally act in different proportions *in vivo*, or perhaps that there is a labile maltase also concerned in the normal processes of digestion.

The presence of a mixture of the two trisaccharides in honeydew might explain the difficulties encountered by Nottbohm & Lucius (1931) in crystallizing melezitose from a sample of *Latschenhonig*. It seems very likely that the high apparent dextrin content of honeydew honeys (cf. Nottbohm & Lucius, 1931) is sometimes due to melezitose (cf. Walton, 1942) and sometimes to the true dextrans and 'dextrin-fructosides' in the 4^g- α -glucosylsucrose type of honeydew (Wolf & Ewart, 1955*a*). It would be interesting, for example, to know whether the honeydew of the scale insect *Lecanium hemicryphum*, which gives a short but abundant flow of honeydew on spruce in early summer, owes its high dextrin content (28 % of its dry wt.; Gontarski, 1940) to the substances here observed with *Eulecanium coryli*.

It is peculiar that among members of the same families (Coccidae: Wolf & Ewart, 1955*b*; Ewart & Metcalf, 1956; Aphididae: present study) there should occur two different types of sucrose digestion. Dr A. Maurizio (of the Bienenabteilung, Eidg. Milchwirtschaftliche Versuchsanstalt, Liebefeld-Bern) informs us that in certain years melezitose honey is found even in the spruce-fir region of Switzerland, whereas in other years the honey from this region, although derived from honeydew, is free from the trisaccharide. This points to the existence of both types of insect on spruce (*Picea excelsa*). She suggests that it would be worth while for entomologists to make a survey of aphid species to find out which of them produce melezitose. Such a survey would not be difficult, because the chromatographic tests described here distinguish the two types of honeydew fairly quickly. The result might be viewed in the light of the present classification of aphids, which seems to be based mainly on their morphology.

If the distribution of the melezitose-forming enzyme should seem to be random with respect to

existing classifications, it may be necessary to consider the intracellular symbionts which are very commonly found in insects (Steinhaus, 1946). The sucrose-splitting enzymes so far studied in fungi and yeasts are transfructosylases, but among bacteria a number of transglucosylases are known (cf. Hehre, 1951).

In any case, further experiments are necessary to locate the enzymes responsible in the body of the insect, and to try to decide whether more than one enzyme is responsible in *E. tiliae* for the formation of melezitose and 4^g- α -glucosylsucrose. These investigations are, however, not likely to alter the main conclusion from the present work: that melezitose in the honeydew of the lime tree is an insect product. Bearing in mind the possibility that symbiotic micro-organisms are involved, one hesitates to infer that melezitose is produced only in the bodies of the insects, but it would seem probable that this is true of all samples so far examined.

SUMMARY

1. The sugars of the leaves of certain trees (*Tilia*, *Fraxinus*, *Larix*) have been examined by chromatographic methods, special attention being paid to the possible occurrence of melezitose and 4^g- α -glucosylsucrose ('maltosyl-fructoside': White & Maher, 1953).

2. The honeydews produced by *Eucallipterus tiliae* L., *Eulecanium coryli* L. and *Psyllopsis fraxinicola* Frstr. have been similarly examined.

3. Although the honeydew of *E. tiliae* was found to contain about 40 % of melezitose (cf. Maquenne, 1893), analyses of the *Tilia* leaves on which it feeds showed the presence of not more than 2.5 mg. of melezitose/100 g. fresh wt.; the leaves contained about 1000 times as much sucrose.

4. Whole *E. tiliae*, when crushed in sucrose solution, brought about its hydrolysis and the concomitant formation of melezitose and 4^g- α -glucosylsucrose.

5. The origin of melezitose is discussed in relation to these and other findings.

We are deeply indebted to the late Mr J. Pryce-Jones, who initiated a correspondence with one of us (J.S.D.B.) in 1951, and lent us his collection of references and other papers relating to the occurrence of melezitose in honey; and also to Dr T. E. Mittler, Department of Zoology, Birkbeck College, for helpful discussions, and information about his own and other work on the problem. We wish to thank Professor E. J. Bourne, then of the Department of Chemistry, Birmingham University, for examining the infrared spectra of melezitose samples; Mr J. P. Doncaster, of the British Museum (Natural History), for the identification of aphids and other insects; Dr Hewitt G. Fletcher, jun., for a gift of authentic melezitose; Mr G. P. Hill, of the Department of Botany, Nottingham University, for samples of willow sap

collected by the technique of Kennedy & Mittler; Mr G. D. Holmes, of the Forestry Commission, Wrecclesham, for seeds of *Larix* spp. and instructions in germinating them; Dr Nagelschmidt and his colleagues of the Safety in Mines Research Establishment, for help in the identification of melezitose (see Addendum); Dr C. D. Pigott, of the Department of Botany of this University, for identifying the samples of *Tilia* leaves used; Mr A. Snoddy, Curator of the Sheffield Botanic Gardens, for permission to collect material; and Mr D. J. Williams, of the Commonwealth Institute of Entomology, for identifying the scale insect. During part of the work technical assistance was provided by the Agricultural Research Council, whose help we gratefully acknowledge.

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ADDENDUM

X-Ray Data for Melezitose

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(Received 23 October 1956)

Hehre & Carlson (1952) have published an X-ray powder photograph of melezitose. Dr Hewitt G. Fletcher, jun., who obtained it, informed Dr Bacon that he did not intend to publish any more detailed information. It was therefore thought useful to present the following results, which confirm and extend his observations.

Hydrated forms of melezitose. Richtmyer & Hudson (1946) were of the opinion that the stable form of melezitose was the monohydrate. They found

that samples of the dihydrate lost weight when left exposed to the air in their laboratory. This loss of weight, which corresponded to the loss of one molecular proportion of water, was rapid with powdered specimens (3 days) but slower with larger crystals (about 2 weeks). A sample of melezitose kindly sent from Dr Hudson's laboratory by Dr Fletcher was essentially the monohydrate ($C_{18}H_{32}O_{16} \cdot H_2O$), 470 mg. losing 20 mg. when heated for 15 hr. at 100° at 0.5 mm. Hg (calc. loss for

conversion of monohydrate into anhydrous form: 16 mg.), and regaining 18 mg. on exposure to the air of the laboratory.

When another sample was recrystallized by dissolving 0.5 g. in 1 ml. of water, warming, adding 3 ml. of absolute ethanol and leaving it to stand overnight, the large crystals obtained corresponded to a dihydrate, 339 mg. losing 23 mg. in 18 hr. at 100° at 0.5 mg. Hg (calc. loss for conversion of dihydrate into anhydrous form: 23 mg.), and retaining 12 mg. on exposure to laboratory air.

The X-ray diffraction patterns of the monohydrate and the dihydrate showed clear differences by which the two substances could readily be distinguished (see below). By using the diffraction pattern as an indication of the state of hydration it was found that crystals of the dihydrate, even when powdered, did not lose water in the air of this laboratory to any significant extent during 3 weeks. However, when placed in a desiccator over CaCl_2 , they were converted into the monohydrate in less than 5 days. A sample of the sugar, recrystallized

from about 90 % ethanol by dissolving 0.1 g. in 0.4 ml. of 50 % (v/v) aqueous ethanol and adding 1.6 ml. of absolute ethanol, was found to be the monohydrate (cf. experiment of Dr W. T. Haskins, quoted by Richtmyer & Hudson, 1946).

It would thus seem that the loss of water from melezitose dihydrate cannot in all laboratories be as fast as that observed by Richtmyer & Hudson in the National Institute of Health, and it may be, for example, that under Tanret's working conditions (Tanret, 1906; see also Hudson, 1946) the dihydrate was as stable as it is under our own.

The anhydrous melezitose takes up water so rapidly that the time taken to transfer the sample into a sealed-glass capillary is sufficient for it to change into the monohydrate. It is probable that an X-ray pattern of the anhydrous melezitose can only be obtained if it is sealed into a glass capillary under vacuum; this has so far not been done.

X-ray data. X-ray powder-diffraction patterns of melezitose dihydrate and melezitose monohydrate were taken with filtered $\text{Co K}\alpha$ radiation

Table 1. *Powder-diffraction data of melezitose monohydrate*

<i>d</i> (Å)	Relative intensities	<i>hkl</i>	<i>d</i> (Å)	Relative intensities	<i>hkl</i>
12.00	0.02	110			{ 221
9.57	0.18	200	4.71	0.32	{ 311
8.10	0.02	210			{ 410
7.66	0.31	020	4.56	0.21	{ 230
		{ 120	4.25	0.05	{ 031
7.12	0.13	{ 101			{ 420
6.82	0.33	011	4.13	0.38	{ 401
6.43	0.08	111	4.06	0.42	{ 330
5.95	0.19	{ 310	3.99	0.03	{ 411
		{ 201			{ 500
5.56	0.24	211	3.90	0.10	{ 231
5.41	0.03	021			{ 040
5.21	0.03	{ 121	3.82	0.01	{ 510
		{ 030			{ 140
4.89	1.00	400	3.71	0.14	{ 102

Table 2. *Powder-diffraction data of melezitose dihydrate*

<i>d</i> (Å)	Relative intensities	<i>hkl</i>	<i>d</i> (Å)	Relative intensities	<i>hkl</i>
12.12	0.13	110			{ 321
9.90	0.19	200	4.17	1.00	{ 131
7.75	0.71	020			{ 420
7.18	0.19	120	4.00	0.12	{ 411
6.75	0.54	011			{ 421
6.42	0.09	111	3.65	0.21	{ 012
		{ 220			{ 331
6.18	0.15	{ 310	3.58	0.41	{ 112
		{ 211			{ 430
5.58	0.69	021			{ 212
5.40	0.20	{ 301	3.43	0.23	{ 511
		{ 400			{ 600
4.95	0.72	{ 221	3.29	0.27	{ 241
		{ 311			{ 302
4.73	0.72	{ 410	3.25	0.37	{ 431
		{ 230			{ 332
4.59	0.10		2.75	0.31	{ 502

($\lambda = 1.790 \text{ \AA}$) on a camera of diameter 114.6 mm. The principal diffraction lines measured, together with their intensities, are summarized in Tables 1 and 2.

Oscillation and Weissenberg photographs of a single crystal of the monohydrate showed the crystal to be orthorhombic, and together with powder data the unit cell is calculated to be $a = 19.62 \pm 0.20$, $b = 15.54 \pm 0.16$, $c = 7.56 \pm 0.07 \text{ \AA}$. The assignment of four molecules to the unit cell gives a calculated density of 1.505 g./cm.^3 compared with a measured density of 1.51 g./cm.^3 (pycnometer method).

X-Ray powder diffraction lines of the dihydrate were indexed on the basis of an orthorhombic cell

with dimensions $a = 19.80 \pm 0.20$, $b = 15.65 \pm 0.16$, $c = 7.51 \pm 0.07 \text{ \AA}$. The observed density (by flotation method) of 1.55 g./cm.^3 (cf. Pionchon, 1897) agrees with the calculated value of 1.54 g./cm.^3 .

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The Preparation and Properties of Ribonucleic Acids from Rat Liver

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Early methods of preparing ribonucleic acid (RNA) from mammalian tissues and yeast involved the use of alkali (Levene & Bass, 1931), but it is now recognized that this process necessarily involves a considerable amount of degradation (cf. Allen, 1954). On the other hand, if very mild conditions are used there is a danger of degradation by ribonuclease during the preparation.

Attempts have been made to inactivate the ribonuclease without affecting the RNA, but none of the substances used seems to be entirely satisfactory. Thus Grinnan & Mosher (1951) used 4-m-guanidine hydrochloride as a denaturing agent, followed by heat to remove the protein. This yielded a product stated to have a sedimentation coefficient $S = 10\text{s}$ corresponding to a high molecular weight (about 260 000). Gulland, Barker & Jordan (1945) have shown, however, that guanidine hydrochloride denatures deoxyribonucleic acid, and the probability of such action upon RNA, followed by aggregation, cannot be overlooked. Kay & Dounce (1953) have used sodium dodecyl sulphate as a protein precipitant and they obtained material in 50% yield having sedimentation coefficients of about 3s, corresponding to a molecular weight value of $2-3 \times 10^4$. It has since been shown by Allen (1954) that dodecyl sulphate does not completely inactivate ribonuclease under the conditions obtaining in this method of preparation. Strong salt will split a certain proportion of the cytoplasmic nucleoprotein, but only on prolonged standing, and

RNA thus obtained by Kerr & Seraidarian (1949) showed evidence of considerable enzymic depolymerization.

In the course of investigating these methods, it was found that after mild acid treatment a large part of the nucleic acid was easily separated from the associated protein and lipid by a short period of heating in salt solution. The protein flocculated and precipitated, leaving most of the nucleic acid in solution together with a small amount of protein which could readily be removed by digestion with chymotrypsin. It was also found that this process is effective with isolated cell fractions. The ribonucleic acids obtained have sedimentation coefficients in the range 2-3s, corresponding to molecular weights of $2-3 \times 10^4$. It is possible that the ribonucleic acids of high molecular weight obtained by some other workers are artifacts produced by aggregation of particles as a result of the reagents employed. It must be admitted, however, that some depolymerization may occur during the heating process.

EXPERIMENTAL AND RESULTS

Preparation of ribonucleic acids from whole cytoplasm. Freshly excised and chilled rat livers were minced and homogenized for 30 sec. in 9 vol. of 0.14M-NaCl in a Waring Blender, and nuclei, cell debris and unbroken cells were removed by centrifuging at 1200 g for 10 min. All operations up to the heating process were carried out below 3° and as quickly as possible to minimize enzymic degradation. The crude nucleoprotein was obtained as a white precipitate by