A Nitrogenous Derivative of Lactose from Lactating Rat Mammary Gland

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During an investigation of the synthesis of lactose by various preparations from rat mammary glands (see Heyworth & Bacon, 1955) a new substance was noticed on paper chromatograms of extracts made in the early stages of lactation. Shortly afterwards Caputto & Trucco (1952) reported the presence in such extracts of several oligosaccharides, one of which they suggested was a tetrasaccharide. We attempted the isolation of the substance, with the possibility in view that it arose as a by-product or intermediate in lactose synthesis. The idea that it was exclusively carbohydrate in nature was weakened by the discovery that acid hydrolysis only doubled its reducing power, and after the report by Leloir & Cardini (1953) that Caputto & Trucco's compound was a nitrogenous derivative of lactose we found that our own partially purified preparations also contained nitrogen. Although our method of purification differs considerably from that used by Trucco & Caputto (1954), the substance obtained resembles their 'neuramin-lactose' in many respects. Our compound, like 'neuramin-lactose' and 'compound E' (Caputto & Trucco, 1952), gives the direct Ehrlich reaction and a purple colour with Bial's reagent, which are characteristic of sialic acid (Blix, 1936) and neuraminic acid (Klenk, 1941). Although we normally used boiling water for its extraction, it may equally well be extracted with cold water. It therefore has a particular interest as a naturally occurring derivative of sialic acid of low molecular weight; hitherto sialic acid has been found only in mucins, glycoproteins and complex lipids.

The substance has no free amino group, and among the products of its alkaline degradation is a substance very similar to, if not identical with, 2-carboxypyrrole (Gottschalk, 1953, 1955*a*). It therefore resembles sialic acid more closely than neuraminic acid (methoxyneuraminic acid: Klenk & Faillard, 1954).

In this paper we describe the method of purification and some of the properties of the compound,

including the products of its acid and alkaline degradation. A preliminary account has already been published (Heyworth & Bacon, 1954).

EXPERIMENTAL AND RESULTS

Extraction and purification of the compound

Starting material. The substance was first observed on a paper chromatogram of an aqueous extract of rat mammary gland made at room temperature in the presence of HgCl_2 (see Heyworth & Bacon, 1955); benzidine-trichloroacetic acid (Bacon & Edelman, 1951) showed a spot of low R_F (about 0.03–0.06 in butanol-acetic acid-water, 4:1:5, by vol.; Partridge, 1948) of comparable intensity to the lactose present. Chromatography of extracts of glands at different stages of lactation showed that the compound of low R_F was present at 4, 5, 7, 10 and 12 days' lactation, but to a much smaller extent at 14 and 17 days. Glands of rats of 4–11 days' lactation were used for subsequent preparations.

The compound was not detected in preparations of guinea-pig mammary gland used for metabolic studies (Heyworth & Bacon, 1955), nor in a boiled and concentrated extract of guinea-pig glands (5 μl . applied to paper, corresponding to 7 mg. fresh wt. of tissue). Similarly, the compound was not detected in a boiled and deproteinized extract of a cow udder (10 μl . applied, corresponding to 8 mg. fresh wt. of tissue).

Extraction and deproteinization. The glands were chopped into boiling water and boiled for a further 10 min. to inactivate enzymes. The extract was cooled and blended, and the solid material separated in a refrigerated centrifuge. The hardened layer of fat was removed, and the residue resuspended in water and centrifuged again. The supernatants were collected and deproteinized; unless deproteinized the extract quickly blocked a charcoal-Celite column. Ethanol (0.5 vol.) and chloroform (0.25 vol.) were added, and the pH was adjusted to 4.5 with acetic acid. After being shaken vigorously the mixture was left overnight in the refrigerator. The white protein precipitate was removed by centrifuging, resuspended in ethanol-chloroform-water mixture and again centrifuged. The supernatants were concentrated under reduced pressure, with a water-bath temperature below 50°. Many extracts were divided into two parts (*a* and *b*, Table 1) before being chromatographed. A single preparation was made in which the initial extraction was carried out with cold water.

Chromatography on charcoal—Celite. This was carried out by gradient elution, as described by Bacon & Bell (1953) and Bacon (1954). Usually 40 g. of charcoal-Celite mixture (1:1) was packed in a glass tube of internal diameter 15 mm.

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and eluted from a 500 ml. water reservoir into which 50% (v/v) ethanol was dropped to replace the effluent.

Columns containing Activated Charcoal (British Drug Houses Ltd.) were used for all the initial separations, and Active Carbon no. 130 (Sutcliffe, Speakman and Co. Ltd., Leigh, Lancs.) for the removal of impurities in the effluent from cellulose columns (Bacon, 1954). The columns were washed with about 1 l. of water overnight before the sugar mixture was added. This reduced the acid content of the effluents from B.D.H. activated charcoal some 40 times; the remaining acidity was neutralized with 0.1N-NaOH. The addition of Na⁺ ions increased the dry weight of the preparations from these columns by 1-4%.

The concentration of sugars in the effluent was sufficient for a direct test on paper chromatograms. In early experiments with B.D.H. activated charcoal there was a satisfactory separation of lactose and the unknown compound, which emerged at about 7 and 16% of ethanol respectively. Later batches of this charcoal failed to bring about any separation, but a sample of the same charcoal 'washed with acid' was found which reproduced the earlier results. The two charcoals were thereafter used in a two-step procedure, by which the two substances were first separated from other constituents of the extract, and then from one another.

Chromatography on cellulose powder. The method of Hough, Jones & Wadman (1949) was used, with the modifications described by Bacon (1954).

Evaporation of effluents. This was carried out at neutral pH, under reduced pressure below 50°, the residue being placed in an evacuated desiccator over H₂SO₄ or CaCl₂ at room temperature for determination of dry weight (Table 1) and for subsequent storage.

Types of preparation. Preparations used for analysis and investigation of chemical and physical properties were of two kinds: (i) those resulting from one or more fractionations on B.D.H. activated charcoal-Celite; (ii) preparations of type (i) further purified by partition chromatography on cellulose powder, followed by gradient elution from Active Carbon no. 130-Celite. Where the type of preparation is not specified it may be taken that both types possess the

properties described. Both types showed only one spot on paper chromatograms sprayed with benzidine-trichloroacetic acid. (Here, and elsewhere, unless otherwise specified, the chromatograms were developed with *n*-butanol-acetic acid-water.) Type (ii) preparations showed only one spot, close to lactose, when run in ethyl acetate-pyridine-water (10:5:6, by vol.) at 30° (Trucco & Caputto, 1954); at 20-22° (solvent homogeneous) the spot was more compact and had an *R_F* value 0.7 times that of lactose.

Properties of the partly purified compound

Bial and Ehrlich tests. Bial's reagent (0.2 ml. of 0.25% orcinol, 0.01% of FeCl₃ in 12N-HCl) gave no reaction with the compound (4 mg. in 0.2 ml.) in the cold, but on boiling gave a stable red colour.

Ehrlich's reagent [0.2 ml. of 3% (w/v) *p*-dimethylamino-benzaldehyde in 6N-HCl] gave a green mixture with the compound (4 mg. in 0.2 ml.) in the cold and a red colour on heating.

Because of the acid content of these reagents it was not possible to spray them on to paper chromatograms and then heat them in a dry oven at 100°. However, it was found possible to steam the sprayed chromatogram. The steaming was carried out at atmospheric pressure on a wood and Perspex frame in an autoclave; the spots spread if too much water condenses on the paper.

On chromatograms of the preparations both the Bial's and Ehrlich's reagents gave purple spots corresponding with the sugar spot located by benzidine-trichloroacetic acid). The sugar and Bial-reacting spots also coincided on a chromatogram of the compound in *isobutyric acid-ammonia* (Krebs & Hems, 1953); (*R_F* 0.16; lactose, 0.20; galactose and glucose, 0.31). In this case the purple spot was not seen on steaming, but appeared after several hours at room temperature.

Absorption curve of the products of Bial's reaction. The reaction was carried out according to the methods of Klenk & Langerbeins (1941) and Werner & Odin (1952), with orcinol purified by treatment with charcoal in boiling

Table 1. *Yields, and reducing sugar and nitrogen contents of various preparations of the nitrogenous compound from rat mammary glands*

Type (ii) preparations were made from type (i) preparations of the same number. Preparation 7a (solid) was prepared by precipitation from a methanol solution with ether. For further details see text.

Tissue taken (g.)	Preparation		Dry wt. (mg.)	Reducing sugar* (% dry wt.)	Nitrogen (% dry wt.)	Total nitrogen in sugar position† (%)	Ratio N:lactose
	(No.)	(Type)					
98	6a	(i)	27	31	—	—	—
	6b	(i)	41	43	3.2	—	1.8
109	7a	(i)	65	34	2.9	—	2.2
	7b	(i)	82	34	2.4	—	1.7
	7b	(solid)	12	41	2.2	—	1.3
73	9a	(i)	75	—	3.6	29	—
	9b	(i)	104	—	2.0	69	—
	9a	(ii)	12	27	1.2	82	1.2
105	11a	(i)	75	—	—	—	—
	11b	(i)	151	—	—	—	—
	11a	(ii)	22	23	1.2	—	1.2
105	12a	(i)	56	—	—	—	—
	12b	(ii)	—	—	—	—	—
	12b	(ii)	71	33	1.6	—	1.2

* Reducing sugar is calculated as though it were lactose.

† On paper chromatograms.

benzene and subsequently crystallized from chloroform. With one type (ii) preparation the absorption curve coincided almost exactly with that given by Werner & Odin (1952) for sialic acid. Other preparations gave more indication of the inflexion near 650 $m\mu$ published by Trucco & Caputto (1954), though in no cases quite so pronounced. It seems possible that pentose, which has an absorption peak in this region after treatment under the same conditions, may be responsible for the inflexion.

Absorption curve of the products of the direct Ehrlich reaction. The reaction was carried out as described by Werner & Odin (1952), with *p*-dimethylaminobenzaldehyde purified by the method of Adams & Coleman (1944). The absorption curve was almost identical with that given by them for sialic acid, having maxima at 530 and 560 $m\mu$, the latter somewhat greater.

Acid hydrolysis. In all cases after acid treatment of the compound chromatograms of the products sprayed with benzidine-trichloroacetic acid showed first the liberation of lactose and then the formation of glucose and galactose. [The untreated substance, like lactose, gave a blue-purple colour with the diphenylamine-aniline-phosphoric acid reagent (cf. Harris & MacWilliam, 1954).] Bial- and Ehrlich-reacting products appeared as purple spots on steaming and the same positions appeared as blue spots after several months' storage on chromatograms sprayed with benzidine-trichloroacetic acid or acid aniline phthalate (Table 2).

A hydrolysate (0.8 mg. in 0.1 ml. heated at 100° with 0.9N-H₂SO₄ for 10 min.) was diluted and passed through a column of De-Acidite E (Permutit Co. Ltd., Gunnersbury Ave., London, W. 4). The effluent was concentrated and applied to a chromatogram. After 90 hr. development separate spots of galactose and glucose were detected. No other reducing substances (Trevelyan, Procter & Harrison, 1950) and no hexosamines (Partridge, 1948) were detected in hydrolysates and there was no reaction on spraying with ninhydrin (0.1% in *n*-butanol) or bismuth potassium iodide (Bartley, 1954).

A chromatogram of preparation 9b (Table 1) contained 69% of the total nitrogen in the sugar position. On acid

hydrolysis (0.3N-HCl, 100°, 10 min., Table 2) this nitrogen was redistributed, only 12% remaining in the original position (R_F 0.00-0.04), the remainder appearing from R_F 0.04 to 0.30. (The nitrogen-assay method is described below.)

Ratio of glucose:galactose. Samples of lactose (5.8 mg.) and of the compound (9.6 mg.) were each boiled in glass-stoppered tubes with 0.5 ml. of N-H₂SO₄ for 1 hr. The hydrolysates were cooled, diluted and passed through 5 g. columns of De-Acidite E. The effluents were concentrated to 0.5 ml. and applied to paper chromatograms (20 μ l. along 7 cm. for estimation, two 5 μ l. guide spots.). The chromatograms were developed with *n*-butanol-pyridine-water (6:4:3, by vol.) for 40 hr. The guide strips were sprayed with benzidine-trichloroacetic acid; only glucose and galactose were detected. The monosaccharides were eluted and the reducing powers measured with correction for paper blanks (Heyworth & Bacon, 1955). The ratio of the reducing powers of glucose:galactose both for lactose and the unknown compound was 1.2:1. It was concluded that there was an equimolecular ratio of the monosaccharide residues in the compound.

Reducing power. The lability of the compound to both acid and alkali made it difficult to decide whether a free reducing group was present. The following results are in favour of the presence of such a group:

(a) A chromatogram of the compound was sprayed with the benzidine-acetic acid reagent of Horrocks & Manning (1949); the formation of a brown spot on heating at 100° was as rapid as with glucose.

(b) Chromatograms of the compound dipped in silver nitrate solution in acetone and sprayed with ethanolic sodium hydroxide solution (Trevelyan *et al.* 1950) gave an immediate dark-brown spot.

(c) Evidence was obtained for the formation of an *N*-benzylglycosylamine derivative under the conditions of Bayly & Bourne (1953). In butanol-ethanol-water-ammonia (40:10:49:1, by vol.) lactose and the compound had R_F values 0.13 and 0.05, and the corresponding glycosylamines had R_F values 0.60 and 0.31 respectively. After spraying the chromatograms with 0.1% ninhydrin in butanol

Table 2. *Products of acid treatment of the nitrogenous compound*

R_F values in butanol-acetic acid-water from a number of experiments are given for spots reacting with benzidine-trichloroacetic acid at once (BT) or after storage (slow BT), with Ehrlich's reagent (E), or with Bial's reagent (BL).

Range of R_F value	Conditions of acid treatment					
	0.3N-HCl; 80°		0.3N-HCl; 100° 10 min.	0.5N-H ₂ SO ₄ ; 80°		N-H ₂ SO ₄ ; 100° 3 min.*
	10 min.	60 min.		10 min.	60 min.	
0.0-0.1	0.06 (BT)	0.06 (BT)	0.05 (BT)	0.07 (BT)	0.07 (BT)	0.07 (BT)
0.1-0.2	0.11 (slow BT)	0.11 (slow BT)	—	—	—	—
	0.11 (BL)	0.11 (BL)	—	—	—	—
	—	0.16 (BT)	0.14 (BT)	—	—	—
	0.17 (slow BT)	0.17 (slow BT)	—	—	0.17 (BT)	0.19 (BT)
0.2-0.3	0.17 (BL)	0.17 (BL)	—	—	—	—
	—	—	0.24 (slow BT)	0.23 (BL)	0.23 (BL)	—
0.3-1.0	—	0.30 (BL)	—	0.25 (E)	0.25 (E)	0.27 (E)
	—	—	—	0.27 (slow BT)	0.27 (slow BT)	0.30 (slow BT)
	—	—	—	—	—	0.63 (E)

* In phenol-water: slow acid aniline phthalate, 0.43; Ehrlich, 0.42, 0.58, 0.70. In ethyl acetate-pyridine-water: slow acid aniline phthalate, 0.52; Ehrlich, 0.56.

and heating for 3 min. at 85° the derivatives appeared as yellow spots, which after 30 min. at room temperature changed to purple. Both the original sugars and the derivatives could be detected by spraying with benzidine-trichloroacetic acid. The greatest conversion of lactose into the benzylglycosylamine (about two-thirds) was obtained by heating the sugar on the chromatogram with benzylamine at 85° for 9 min. (instead of 5 min.). The conversion of the compound into the benzylglycosylamine under these conditions was, however, only about one-fifth.

When the compound and lactose were treated with benzylamine on chromatograms and these were developed in butanol-acetic acid, the acidic nature of the solvent caused a breakdown of the benzylglycosylamines and the original sugars appeared unchanged. This suggests that the reaction with benzylamine is reversible, as would be expected if the compound possesses a free reducing group.

The reducing power of the preparations under alkaline conditions was compared with that of lactose by the procedure of Nelson (1944) and Somogyi (1945). A boiling time of 15 min. was sufficient for complete reduction. The determinations were made in duplicate. The percentage of lactose in the compound was calculated on the assumption that the reducing power of the compound is equal to that of its lactose content. The results (Table 1) suggest that lactose accounts for about one-third of the dry weight of the type (ii) preparations.

Increase in reducing power on hydrolysis. The compound was hydrolysed by boiling with $\text{N-H}_2\text{SO}_4$ for 2 hr. The solution was cooled and neutralized with N-NaOH and reducing power measured by the Nelson-Somogyi procedure. The increases in reducing power in two experiments were 70 and 71 % respectively of the reducing power before hydrolysis. A similar increase was found for the hydrolysis of lactose under these conditions.

Nitrogen determinations. Estimations of nitrogen were made on 4 mg. samples of the compound (in 0.5 ml.) by the micro-Kjeldahl procedure. A.R. sulphuric acid (1 ml.) and about 250 mg. of catalyst (5 g. of K_2SO_4 ; 1 g. of CuSeO_3) were added and the mixture was heated for 2 hr. in Pyrex tubes. Determinations and blanks were carried out in duplicate; the results are given in Table 1.

In order to determine how much Kjeldahl nitrogen was associated with the sugar-reacting component of the preparations, and the fate of this nitrogen after hydrolysis, estimations were carried out on strips cut across paper chromatograms.

The compound (about 8 mg. in 200 $\mu\text{l.}$) was applied along 7 cm. of the base-line of chromatograms. The papers were developed for short periods (5–15 hr.) with butanol-acetic acid, or propanol-ethyl acetate-water (7:1:2, by vol.). After drying, successive strips about 2 cm. deep were cut from the paper, and each was incinerated for about 3 hr.

In preparation 9a (Table 1) 29 % of the nitrogen was associated with the sugar. After purification on a cellulose column and on Active Carbon no. 130 the percentage of nitrogen in the sugar position was 82, the remainder being distributed down the chromatogram in amounts no greater than the experimental variation of blank determinations.

Ratio of nitrogen:lactose. From the nitrogen and reducing-sugar estimations the ratio atoms of nitrogen:molecules of lactose was calculated for a number of preparations (Table 1). The preparations which had been purified on cellulose and Active Carbon no. 130 gave consistently the

lowest ratio of 1.2:1. The evidence thus suggests that the ratio of nitrogen:lactose in the compound is 1:1. If the reducing power of the compound is less than that of the corresponding amount of lactose, then the ratio of nitrogen:lactose would be nearer to unity.

Tests for nitrogenous substances. (a) Biuret: a biuret test was negative.

(b) α -Amino acids: chromatograms were sprayed with 0.1 % of ninhydrin in butanol (Consden, Gordon & Martin, 1944) and heated at 95° for a few minutes. Some preparations showed slight contamination with ninhydrin-reacting substances. Preparation 9a (Table 1) showed ninhydrin-positive spots on a butanol-acetic acid chromatogram at R_F 0.16, 0.20 and 0.63 (sugar at R_F 0.03). The ninhydrin-reacting substances were removed by chromatography on cellulose and Active Carbon no. 130.

(c) Determination of amino groups: no nitrogen was liberated by nitrous acid (Van Slyke, 1929) from a type (ii) preparation of the compound (6.2 mg. in 5 ml.).

(d) Basic nitrogen: chromatograms of the compound gave no reaction with the bismuth potassium iodide spray of Bartley (1954).

(e) Hexosamine and *N*-acetylhexosamine: chromatograms of the compound gave no reaction with the sprays of Partridge (1948). The conditions of the Morgan & Elson (1934) determination of acetylhexosamines (0.05 $\text{N-Na}_2\text{CO}_3$ at 100° for 5 min., and treatment with Ehrlich's reagent in the cold) gave no coloration.

Specific rotation. The specific rotations of four type (i) preparations of the compound were measured in a 2 dm. polarimeter tube of about 3 ml. capacity. The values of $[\alpha]_D$ ranged from +6 to +22°.

Ultraviolet absorption. Paper chromatograms of the compound showed no darker areas under u.v. light (Chromatolite Lamp, Hanovia Ltd., Slough, Bucks). The absorption curve of an aqueous solution (preparation 7b, 1.2 mg./ml.) showed very little absorption between 420 and 340 $\text{m}\mu$, rising to an optical density (1 cm. cell) of 0.3 at 250 $\text{m}\mu$, with no peaks.

Phosphate, sulphur, halogen. Phosphate determinations (Berenblum & Chain, 1938) on some preparations of the compound showed traces of phosphate. A type (ii) preparation contained 0.01 % of phosphate. No sulphur or halides were detected in Lassaigne sodium-fusion tests.

pH Titration curve. The compound (15 mg.) was dissolved in water (1 ml.) and titrated with HCl or NaOH from a 0.1 ml. ultramicroburette (The Emil Greiner Co., 20–26 N. Moore St., New York 13, U.S.A.). A Doran hanging-drop electrode was used for the measurement of pH. The curves obtained suggested the presence of a group with pK 2.2, and possibly another group with pK above 12. There was an indication that some change occurred at pH 12, rather more acid being required to reverse the titration than alkali originally consumed.

Paper electrophoresis. Solutions of the compound (3 $\mu\text{l.}$ spots) were applied to a line drawn across the middle of 34 cm. $\times$ 5 cm. strips of Whatman 3MM paper. A 3 $\mu\text{l.}$ spot of 2 % (w/v) lactose was applied to each strip as a neutral marker. The apparatus used was the EEL paper-electrophoresis bath (Evans Electroselenium Ltd., Harlow, Essex). After a few hours with a current of 2 ma the strips were removed and allowed to dry, stretched horizontally on a stainless-steel frame. Lactose always moved towards the cathode by electro-endosmotic flow and its position was

[illegible]

5 hr.). This product also gave a dark area when chromatograms were observed under u.v. light. In an estimation of nitrogen on the 5 hr. chromatogram, 50% of nitrogen appeared at R_f 0.10 and only traces at 0.33 and 0.85.

N-Acetylglucosamine heated at 80° with 0.25N-NaOH gave Ehrlich-reacting products with R_f 0.50, 0.61 and 0.74 at 5 min. (cf. Kuhn & Krüger, 1956). These had disappeared after 20 min. Glucosamine decreased to about one-tenth under the same conditions in 1 hr. With 0.025N-NaOH at 80° the two sugars decreased gradually over 1 hr.

Incubation with enzyme preparations. The compound was incubated at 37° and pH 5 with an almond-emulsin preparation, with a commercial lactase preparation (L. Light and Co. Ltd.) and with a dialysed aqueous extract of lactating rat mammary gland (Heyworth & Bacon, 1955), all of which hydrolysed lactose. Paper chromatography showed no change in the compound.

Incubation with respiring mammary-gland preparations. The compound (5 mg. in 4 ml.) was incubated with respiring slices, blends or mitochondrial suspensions from guinea-pig mammary gland (Heyworth & Bacon, 1955). There was no change in the appearance of the compound on paper chromatograms nor in the reducing power of the lactose position.

DISCUSSION

The partly purified compound here described was obtained in larger amounts per rat than the 'neuramin-lactose' of Trucco & Caputto (1954): 11 mg./rat, or 1.6 mg./g. of fresh tissue, as compared with the 0.4 mg./g. of fresh tissue reported by them. This may be due to the fact that our rats were at an earlier stage of lactation: 4–11 days, as against 15–19 days.

It is difficult to assess the purity of our preparations. Although no other carbohydrate was detectable by paper chromatography in the products of primary fractionation on charcoal, after purification on cellulose columns the material had a rather lower lactose content (as measured by reducing power) than Trucco & Caputto's. Assuming the non-lactose portion of the molecule to be similar to sialic acid ($C_{13}H_{23}O_{11}N$ or $C_{11}H_{19}O_{10}N$) one might expect the lactose to constitute between 49 and 53%; Trucco & Caputto (1954) found values of about 40–42%. It would probably be safe to assume that our best preparations were about 50% pure. On paper chromatography or electrophoresis a typical purified preparation showed a single spot which gave reactions both for reducing sugar and for sialic acid. After acid treatment this was no longer the case, the reducing sugar now having the R_f values of lactose, galactose and glucose (cf. Trucco & Caputto, 1954), and the sialic acid-like material migrating to other regions of the chromatogram. The quantitative data presented here are consistent with the hypothesis that the substance, like 'neuramin-lactose', is composed of one molecule of lactose and one of the nitrogenous sialic acid.

Our purified preparations were homogeneous in

the ethyl acetate-pyridine-water solvent which Trucco & Caputto (1954) used to separate 'neuramin-lactose' from 'compound E', but we have not been able to decide whether it is identical with one or other of these (as seems likely), because Dr Trucco informs us that they have found considerable variations in R_f values, which they attribute to variations in the samples of commercial pyridine that they use.

The name 'neuramin-lactose' was fully justified by the evidence that was available to Trucco & Caputto in 1954, but it would seem from the fact that our compound yields 2-carboxypyrrole on alkaline degradation that it would be more correct to regard it as a sialic acid derivative (cf. Gottschalk, 1956). If, like bovine sialic acid (Blix, Lindberg, Odin & Werner, 1955), the lactose derivative of mammary gland has two acetyl groups, one more labile than the other, it seems possible that 'neuramin-lactose' and 'compound E' differ by one acetyl group. Unfortunately the greater part of the present work was completed in 1954, and we see no opportunity of investigating the acetyl content of our derivative, although it would clearly be very desirable to do so.

The recognition that the lactose derivative contains a substance of the sialic acid type does not help very greatly in understanding its structure, because the structure of sialic acid is still in doubt. The recent formulation of Gottschalk (1955*b*) (cf. Blix, 1956) would seem to provide only one functional group ($>C=O$) by which sialic acid could be attached to lactose, leaving the reducing group of the latter free, although there are evidently several hydroxyl groups through which the lactose could form a glycosidic linkage to the acid (Blix *et al.* 1955). The evidence presented here is not strong enough to decide between these two types of structure. Our evidence for a free reducing group could conceivably refer to the reducing properties of sialic acid itself [but see Gottschalk (1956) for the reducing properties of combined sialic acid] and the failure of three lactase preparations to attack the compound must be used with great caution as evidence against a lactoside structure, in view of the fact that *N*-acetyl-lactosamine is not attacked by emulsin nor by the lactase of *Escherichia coli* (Kuhn & Kirschenlohr, 1954).

The discovery by Dr M. Shilo that cell-free extracts of certain bacteria will liberate lactose from our compound should help considerably towards the identification of the non-lactose moiety, and its point of attachment (see Addendum).

As to the metabolic significance of the compound, Caputto & Trucco (1952) failed to observe any conversion of their substance into lactose, and our experience has been similar. The most promising system in which to test this further would be a

homogenate capable of lactose synthesis, such as those prepared by Reithel, Horowitz, Davidson & Kittinger (1952) and Kittinger & Reithel (1954). We have not been able to obtain such preparations (Heyworth & Bacon, 1955).

From the occurrence of sialic acid in conjugated proteins (including those of colostrum, Kuhn & Brossmer, 1954) one might argue that the presence of the lactose derivative in mammary gland is more likely to be connected with the elaboration of glycoproteins than with the synthesis of lactose. Nevertheless, it is tempting to speculate that the ability to synthesize lactose has been acquired by mammals by the modification of a synthetic mechanism which in other vertebrates is concerned only with the formation of mucins and other glycoproteins. The occurrence of lactose in plants (cf. Reithel & Venkataraman, 1956) is perhaps not relevant to this speculation.

SUMMARY

1. A nitrogenous derivative of lactose was extracted from the mammary tissue of rats of 4–11 days' lactation, and partly purified by gradient elution from charcoal-Celite and by partition chromatography on cellulose powder.

2. The compound gives purple colours characteristic of sialic acid when heated with the reagents of Ehrlich or of Bial, thus resembling the 'neuramin-lactose' of Trucco & Caputto (1954).

3. It further resembles 'neuramin-lactose' in the following: it yields lactose on acid hydrolysis, has a ratio of nitrogen:lactose (by reducing power) of 1:2:1, and has a strongly acidic group (pK 2.2), which the infrared absorption spectrum suggests is in the form of an ionized carboxyl group.

4. The compound differs from neuraminic acid in having no free amino group, and in yielding 2-carboxypyrrole on heating in alkaline solution.

5. It was not attacked by three lactase preparations, nor by respiring slices, homogenates and mitochondrial suspensions from guinea-pig mammary gland.

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ADDENDUM

The Breakdown of the Lactose Derivative by Bacteria

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In view of the lability of sialic acid under acid and alkaline conditions an enzyme preparation was sought that would split the linkage between lactose and the rest of the molecule, preferably without any further degradation of the latter. The action of micro-organisms and enzyme preparations from them was tested by incubating them with type (i) or (ii) preparations of the lactose derivative, and following the course of the reaction by paper chromatography.

RESULTS

Lactose-fermenting yeasts. Washed cells from cultures of lactose-fermenting yeasts supplied by Dr M. Aschner (*Oidium lactis*, *Candida subtropica*, *C. krusei* and *Torulopsis glabrata*) incubated with 20 mg./ml. of type (i) preparation at 30° did not attack the compound. However, bacterial contamination of some of these incubation mixtures led to its rapid breakdown. (For convenience, without prejudice to the final identification of the lactose derivative, these are referred to as 'neuramin-lactose-splitting bacteria'.)

Isolation of neuramin-lactose-splitting bacteria. Solutions of type (i) preparations (20 mg./ml.) were incubated at 30° for 24–48 hr. under non-sterile conditions, with and without the addition of 1% Tryptone (Oxo Ltd., Thames House, London, E.C. 4). From four such incubations active strains were isolated by plating on nutrient agar; all appeared to be identical. They were Gram-negative rods, and the colonies on nutrient agar were large, opalescent and strongly fluorescent under u.v. light. They were subcultured on nutrient agar (Marmite, 0.3%; tryptone, 1%; glucose, 1%; K_2HPO_4 , 0.3%; NaCl, 0.5%; agar, 2%; water 100).

Action of resting cells. The bacteria were grown for 24 hr. on agar slopes of the above composition, washed off with 10 ml. of saline, centrifuged, washed with a further 10 ml. of saline and the centrifuged cells transferred to solutions (20 mg./ml.) of type (ii) preparations. Complete breakdown took place in 12–24 hr., yielding one substance with the

R_f of lactose, and another spot which was Ehrlich-positive. The activity of a culture grown on nutrient-agar slopes to which 1% of hog gastric mucin had been added was not noticeably greater.

Action of cell-free extracts. Cells were grown and harvested as described above. The thick paste of cells obtained on centrifuging was cooled to -15° and crushed in a Hughes press (1951). The cell-free extract was separated by centrifuging (about 10 000 g); when stored frozen it retained its activity for several weeks. This extract (0.1 ml.) brought about the complete breakdown of type (ii) preparations (0.3 ml., 4 mg./ml.) in 4–9 hr. in the presence of chloroform, but many unwanted substances were simultaneously produced from the extract itself. All the side reactions detectable by the spraying agents used were eliminated by dialysis of the extract for 48 hr. against distilled water at 4°.

Properties of the breakdown products. The sugar released from combination had the same R_f as lactose in four solvent systems: *n*-butanol-acetic acid-water (4:1:5, by vol.), ethyl acetate-pyridine-water (2:1:2, by vol.; Zilliken, Smith, Rose & György, 1954), *n*-butanol-ethanol-water-ammonia (40:10:79:1; Bayly & Bourne, 1953) and ethyl acetate-*n*-butanol-water. The addition of a lactase preparation (L. Light and Co., Colnbrook, Bucks.) brought about the complete hydrolysis of the sugar and the appearance of two sugars with the R_f values of galactose and glucose.

The only other product detected was a Bial- and Ehrlich-positive spot, with an R_f rather greater than that of lactose in butanol-acetic acid-water (lactose has R_f about 0.08 on Whatman no. 1 paper), but hardly moving from the base line in ethyl acetate-butanol-water or in butanol-ethanol-water-ammonia. Its behaviour in butanol-acetic acid-water is thus distinguishable from that of acetylneuraminic acid (R_f 0.27 on Schleicher & Schüll 2043b paper, Klenk & Faillard, 1954), of lactaminic acid (R_f 0.85 with *sec*-butanol, Kuhn & Brossmer, 1954), of gynaminic acid (R_f 0.66, Zilliken, Braun & György, 1955) and probably of methoxynneuraminic acid (R_f 0.72, Kuhn & Brossmer, 1954; 0.72, Zilliken *et al.* 1955; but 0.079, 0.084, Klenk & Faillard, 1954). This spot also gave a blue reaction after 3–4 weeks on chromatograms

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sprayed with benzidine-trichloroacetic acid. On some occasions the treatment with Bial's reagent seemed to show two very closely situated spots, but it is possible that this was a result of the migration which tends to occur during the steaming of the sprayed chromatogram.

Effect of pH. This was not investigated systematically, but the activity of cell-free extracts was distinctly greater at pH 5 than at higher pH values (up to 7.7). Sodium acetate-acetic acid buffer (0.05M, pH 5.0) was added to incubation mixtures in the later experiments.

Neuramin-lactose splitting enzymes in other bacteria. (a) *Pseudomonas* spp. Cultures of *P. fluorescens*, *P. stutzeri*, and *P. pyocyaneus* were kindly provided by Dr S. R. Elsdon of the Department of Microbiology of this University. Grown on the medium described above, and harvested and crushed in the same way, all three bacteria yielded cell-free extracts capable of splitting the lactose derivative, although more slowly than that from the strain first isolated; the extracts did not attack lactose.

(b) *Lactobacillus bifidus*, strain E, requiring the *bifidus* factor for growth, was very kindly provided by Dr D. J. D. Hockenfull, of the Glaxo Research Laboratories. It was grown in a liquid Boventer liver-infusion medium supplemented with 1% Bactopectone (Difco Laboratories, Detroit, U.S.A.), 0.5% NaCl, 0.01% cystine, 1% lactose and 10% fresh deproteinized human milk (Tomarelli, Norris & György, 1949). The pH was adjusted to pH 7.4 and 400 ml. portions were sterilized in 500 ml. conical flasks in an autoclave (15 lb./in.² for 15 min.). After inoculation with 5 ml. of a 24 hr. culture of the organism in the same medium, the flasks were incubated for 48 hr. at 37°. The cells were harvested and crushed as before.

Washed-cell suspensions and cell-free extracts split the lactose derivative, giving the Ehrlich- and Bial-positive substance described earlier, but no lactose; instead, monosaccharide appeared. The cell-free extract had lactase activity, which in the presence of *N*-acetylglucosamine produced a series

of oligosaccharides similar to those described by Zilliken *et al.* (1954).

In the hope of inactivating the lactase the cell-free extract was dialysed for long periods against distilled water. When the lactose derivative was incubated with extracts dialysed for 18–24 hr., lactose was formed, indicating that the initial step in the action of extracts of *L. bifidus* is similar to that seen with the other bacterial extracts.

It would therefore seem that the capacity to split compounds like neuramin-lactose is of fairly widespread occurrence among bacteria.

SUMMARY

1. Washed-cell suspensions and cell-free extracts of several species of bacteria have been found to attack the lactose derivative from mammary gland described by Heyworth & Bacon (1957).

2. Two products of this action have been detected: a sugar having the chromatographic behaviour of lactose, and a sialic acid-like substance distinguishable from lactaminic acid (Kuhn & Brossmer, 1954) and gynaminic acid (Zilliken *et al.* 1955), and probably from acetyl- and methoxy-neuraminic acids (Klenk & Faillard, 1954).

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