haemochromogens and monocyan-haem, but shows a striking resemblance to those of dicyan-haem and dicarbylamine-haem.

6. The bearing of the similarity of the absorption spectra of dicarbylamine-haem, dicyan-haem and dihydroxyl-haem on the structure of this compound is discussed.

7. Dihydroxyl-haem, unlike dicyan-haem, is paramagnetic, and when treated with carbon monoxide gives CO-haem.

8. The knowledge of the absorption spectrum and other properties of the dihydroxyl-haem compound is of special importance for the study of the reactions between haems and nitrogenous compounds.

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The Semi-micro Estimation of Lactose Alone and in the Presence of Other Sugars

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In the course of studies on the intermediary metabolism of the mammary gland, and in particular the investigation of lactose formation in incubated tissues, it became necessary to devise improved methods for estimating this sugar in the presence of glucose and glycogen. The methods hitherto available for the estimation of small amounts of lactose, alone or in the presence of other sugars, are laborious and unsatisfactory. Those normally used for estimating lactose in milk suffer from a lack of specificity which makes them valueless in any investigation where mixtures of sugars and related carbohydrates are likely to be found, while those designed for more general use rely on the complementary techniques of yeast fermentation, for removing fermentable sugars, and the use either of reduction methods or of a lactose-fermenting yeast to give the lactose equivalent of the residual fermented solutions (Grant, 1935; Scott & West, 1936). The application of paper chromatography to the separation and characterization of sugars in mixtures (Partridge, 1946, 1948), and the adaptation of this method to the estimation of sugars (Flood, Hurst & Jones, 1947; Hawthorne, 1947), are advances of great value, but the techniques are time-consuming and, for other reasons, frequently unsuited for routine work (see Westall, 1948).

In the hope of obtaining a method which would be more convenient in practice and yet have greater specificity than those ultimately dependent on estimations of reducing power, the colour reaction given by lactose with methylamine in alkaline solution, first observed by Fearon (1942) and later used in qualitative studies for detecting lactosuria in pregnancy and the early puerperium (Harwood, 1946; Archer & Haram, 1948), has been developed into a quantitative method. Fearon found that the

reaction was not given by other sugars (except maltose), or by many other substances, including lactic and pyruvic acids, normally associated with carbohydrate metabolism. Since maltose can readily be removed by fermentation the reaction clearly offered the possibility of a specific estimation for lactose which might satisfy an urgent need in studies on the metabolism of the mammary gland.

EXPERIMENTAL

Reagents

Lactose. Pure bacteriological (Kerfoot).

Methylamine hydrochloride. Prepared from 40% methylamine in water (L. Light and Co., Ltd.) by neutralizing with HCl, evaporating the solution to dryness on a water bath, extracting with and recrystallizing from ethanol.

Zinc hydroxide. Prepared from zinc acetate by the method of Letonoff (1934).

Estimation of lactose alone in aqueous solution

The solution (8 ml. containing 4–16 mg. lactose) is pipetted into a boiling tube (6 × 1¼ in.) and treated with 6 drops (0·32 ml.) 10% (w/v) methylamine-HCl solution. The tube is loosely stoppered with a glass cap and the mixture heated in a boiling water bath. After heating for 7 min. and 5 sec., 0·4 ml. of 4·4N-NaOH are added, and 10 sec. later the tube is removed from the bath and the contents mixed and allowed to cool in air. The red colour develops in the stoppered tube during the course of the next 2 min., and is measured in a Spekker photoelectric absorptiometer using a spectrum green filter (Ilford no. 604, 520 mμ.) in conjunction with heat-absorbing filters (Hilger H 503). The solutions are read against water in the second cell and with the drum setting initially at unity.

To ensure maximum colour development the estimations are carried out with strict adherence to a time schedule. They may be done conveniently in an overlapping series, when 6 or 7 may be completed in 1 hr. The full time scheme is as follows for the first two members, *A* and *B*, of a series:

0 sec.	Add 6 drops 10% methylamine-HCl to *A*.
15 sec.	Place *A* in boiling water bath and insert stopper.
7 min. 20 sec.	Add 0·4 ml. 4·4N-NaOH to *A*.
7 min. 30 sec.	Remove *A* from bath.
9 min. 0 sec.	Add 6 drops 10% methylamine-HCl to *B*.
9 min. 15 sec.	Place *B* in boiling water bath and insert stopper.
9 min. 30 sec.	Filter *A* directly into absorptiometer cell. (This is unnecessary in the case of simple aqueous solutions, but essential when salt solutions containing Ca^{++} or Mg^{++} are used, or when the estimations are on biological extracts).
10 min. 45 sec.	Measure *A* in absorptiometer.
16 min. 20 sec.	Add 0·4 ml. 4·4N-NaOH to *B*, and continue as outlined above.

Under these conditions the calibration curve for standard lactose solutions is linear over the range 4–16 mg. (0·05–0·2%); below this range the increase in the colour with increase in concentration is less pronounced, and the method, although useful, is decidedly less accurate for solutions between 0·025 and 0·05% (Fig. 1).

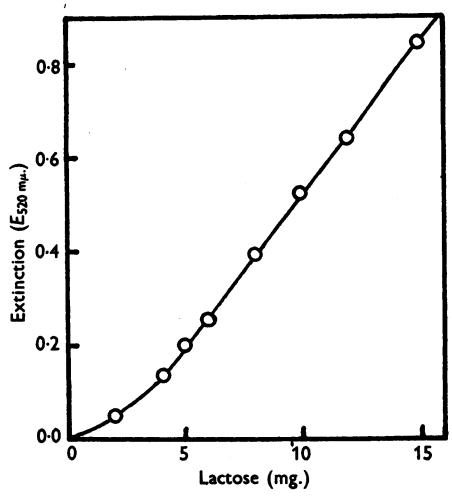

Fig. 1. Calibration curve for standard lactose solutions.

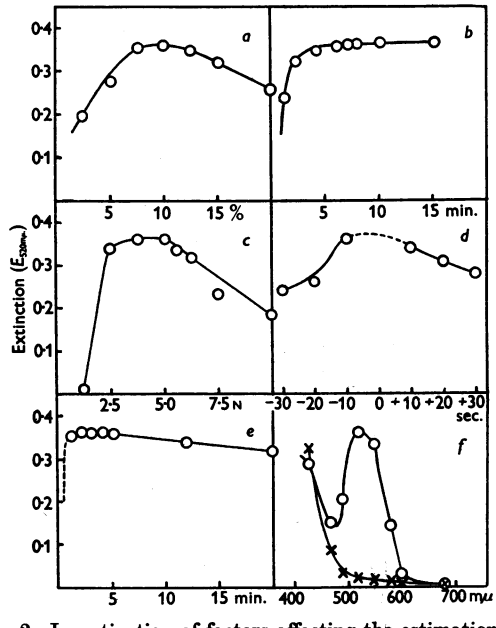

Fig. 2. Investigation of factors affecting the estimation of lactose (0·1% (w/v); cell 1 cm.); (*a*) strength of methylamine-HCl solution; (*b*) time of heating; (*c*) strength of NaOH; (*d*) time of addition of NaOH relative to removal from the water bath; (*e*) rate of development of colour after removal from the water bath; (*f*) absorption spectra of lactose compound O——O and glucose compound ×——×.

The results of an investigation into the optimum conditions of the reaction are given in Fig. 2. The effects of altering the strength of the methylamine-HCl solution (*a*),

the time of heating (*b*), strength of NaOH (*c*), time of addition of NaOH relative to the time of removal from the water bath (*d*), and the time of estimating after removal from the water bath (*e*) are shown, together with the visible absorption spectrum of the lactose compound (*f*). The first, third and fourth of these possible variants are clearly critical and must be strictly controlled. In the further work to be discussed in this paper 5N-NaOH was used; from a reassessment of the method, however, it would seem preferable to use a slightly weaker solution and 4·4N-NaOH is now recommended. The choice of the time for adding the NaOH relative to removing the tube from the water bath necessarily involves considerations of technical convenience, and although there are indications, both from experiment and

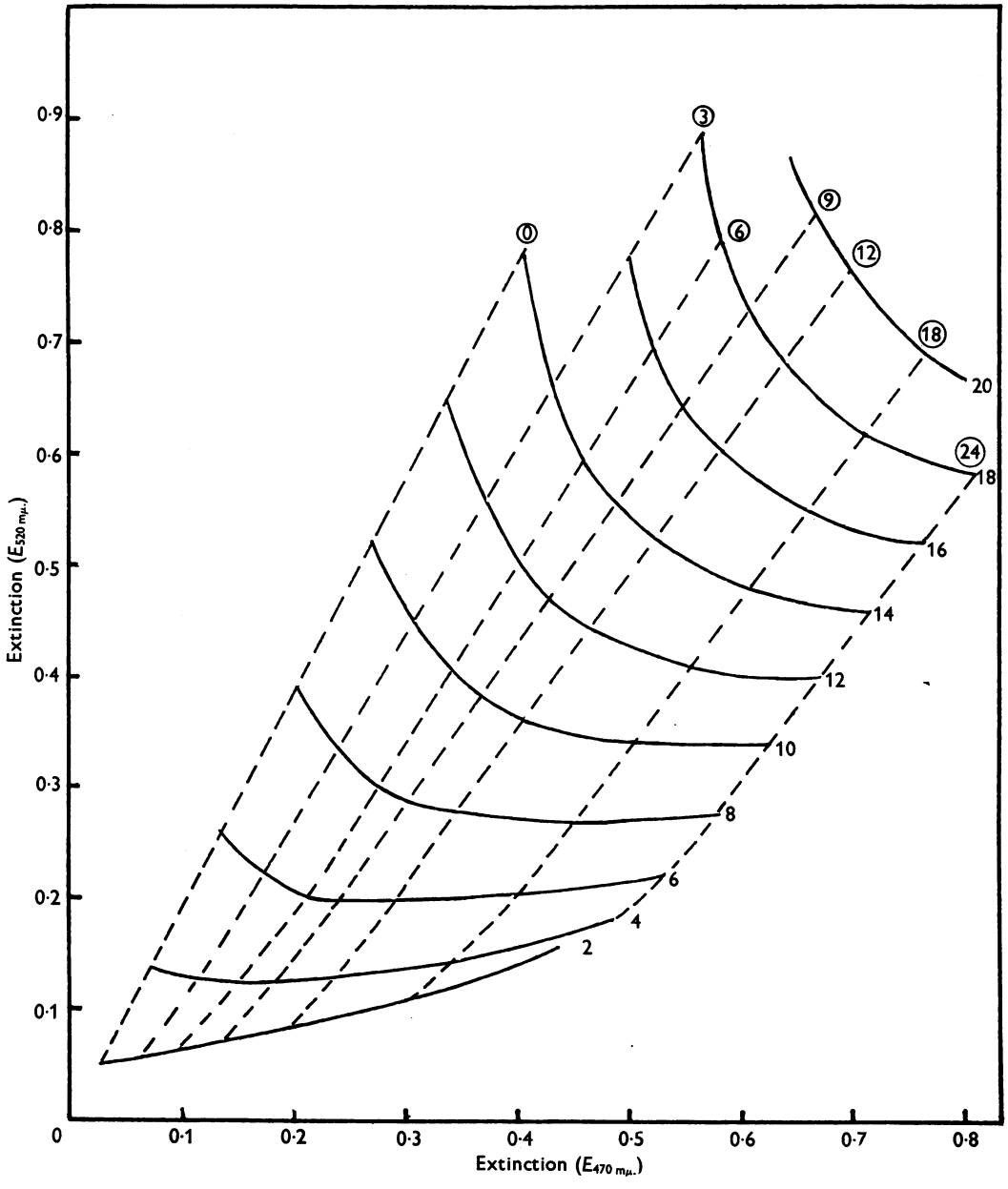

Fig. 3. Composite graph used in estimating lactose in the presence of glucose: ——, curves for constant lactose concentration, equivalent (mg. lactose) given in plain numbers; - - - -, curves for constant glucose concentration, equivalent (mg. glucose) given in 'circled' numbers.

from an interpolation of partial curves (*d*), that the extinction values would be slightly higher if the removal coincided with the addition of NaOH, the small gain in sensitivity would be more than offset by the manipulative inconvenience.

Estimation of lactose in the presence of glucose in aqueous solution

When estimated in the same way, glucose gives a yellow colour instead of red, and it is therefore possible to estimate lactose in the presence of glucose, without preliminary fermentation, by measuring the optical density at two wavelengths. The additional wavelength selected (470 mμ., Ilford no. 602, used in conjunction with heat-absorbing filters, Hilger H 503) gives appreciable, but suboptimal, absorption for glucose alone (see absorption spectrum, Fig. 2(*f*)), and coincides approximately with a point of relatively low absorption for lactose.

From the results given by a series of standard mixtures covering the range lactose 0–20 mg., glucose 0–24 mg., curves are drawn, by plotting the extinction at 520 mμ. against the extinction at 470 mμ., relating these values to constant lactose concentrations measured in the presence of varying glucose concentrations (Fig. 3). From these curves lactose values may be read directly for any pair of extinction values. The method introduces a source of subjective error in the need for interpolating values between the standard curves thus obtained. Interpolation is made on lines parallel to the transverse curves (broken lines in Fig. 3) connecting points of the same glucose content on the different lactose curves. These transverse curves clearly allow the simultaneous determination of glucose in the solutions by a similar process of interpolation, this time along lines parallel to the standard lactose curves. The following examples may be demonstrated on Fig. 3.

Example I. Extinction 520 mμ. = 0·200; extinction 470 mμ. = 0·240. These readings give a point on the intersect of the 6 mg. standard lactose curve and the 9 mg. transverse glucose curve, and indicate a solution containing 6 mg. lactose and 9 mg. glucose.

Example II. Extinction 520 mμ. = 0·272; extinction 470 mμ. = 0·238. These readings involve interpolation for both the lactose and the glucose values, and give the result, 7·2 mg. lactose and 5 mg. glucose.

Sensitivity of the method

For simple solutions of lactose the accuracy of the method in the most favourable range (0·05–0·2%) falls well within ±5%. In the presence of glucose the accuracy varies with the relative amounts of the two sugars, and the direct application of the method is not recommended, except for qualitative work, for solutions containing less than 0·05% lactose, or for any solution containing more than 0·15% glucose. In such cases preliminary fermentation should be carried out and the lactose determined alone in the fermented solution. It is our experience that in the range suggested here lactose can be estimated with an accuracy of ±5%; the glucose value is much less accurate (Table 1).

Estimation of lactose in the presence of glycogen

Solutions of glycogen give a strong opalescence when treated according to the standard method, and it is essential to remove glycogen before the estimation. This is done by precipitation with an equal volume of ethanol. After standing for 5 min. the solution is filtered and suitable samples of the ethanolic filtrate transferred to boiling tubes; the solutions are evaporated to dryness on a water bath, the residue dissolved as far as possible in 8 ml. distilled water and the estimation carried out as in the standard method. If salts are present it is necessary to adjust the pH of the solutions to between 7·0 and 7·4 before drying, in order to prevent decomposition or charring of the sugar.

Table 1. *Recoveries of lactose from lactose-glucose solutions of varying composition*

Lactose present (mg.)	Glucose present (mg.)	Lactose found (mg.)	Glucose found (mg.)	Lactose error (%)
1·8	0·6	1·6	0·9	−11
1·8	1·8	1·6	2·2	−11
1·8	6·0	1·6	6·0	−11
2·4	1·2	2·2	1·9	− 8
2·4	2·4	2·3	2·7	− 4
2·4	6·0	2·0	6·3	−17
3·0	12·0	2·9	11·2	− 3
3·0	15·0	3·0	13·7	0
6·0	9·0	6·0	7·0	0
6·0	24·0	6·0	20·0	0
18·0	6·0	16·8	2·2	− 7
18·0	12·0	17·6	8·5	− 2

Estimation of lactose in solutions containing soluble nitrogen (tissue 'incubates') and salt mixtures

Colour development is partially inhibited in tissue 'incubates' unless such solutions are previously deproteinized. The use of strongly acid or alkaline reagents for deproteinization would clearly complicate the subsequent estimation, and in the search for a neutral precipitant zinc hydroxide (Letonoff, 1934) was tried and found most satisfactory. It has the additional advantage that its use involves no alteration in the volume of the solutions treated and no diminution of the lactose content. Approximately 0·1 g. $Zn(OH)_2$ is added/30 ml. of solution, and the mixture is allowed to stand for 5 min. and then filtered. The filtrate is used for the estimation without further treatment. The addition of $Zn(OH)_2$ may be omitted when glycogen is present, since the ethanol also precipitates the protein.

Solutions pre-treated with $Zn(OH)_2$, or solutions containing Ca^{++} or Mg^{++} (e.g. Ringer-phosphate solution), give a precipitate of insoluble hydroxides when NaOH is added during the estimation. In such cases the solutions are filtered through Whatman no. 1 filters at 9 min. 30 sec. (see time scheme for method).

DISCUSSION

We have extended Fearon's (1942) observations on the specificity of the lactose-methylamine reaction and find that, in addition to maltose, cellobiose and certain lower dextrins give a similar colour. The dextrins are only slowly fermented by brewer's yeast and are thus readily distinguishable from maltose which is rapidly fermented. A limited investigation of their reduction values before and after acid hydrolysis, using the method of Somogyi (1945), gave ratios of approximately 1/5. It seems probable, therefore, that the specificity of the

reaction extends to disaccharides and lower polysaccharides containing the 1:4 glycosidic linkage, irrespective of their stereochemical relationships. In agreement with this, sucrose (1:2) and melibiose (1:6) fail to give the reaction, as do the trisaccharides raffinose and melezitose.

The method seems to offer scope for further application to mixtures of lactose with other sugars, either by direct measurement—when the contaminating sugar gives no colour (raffinose)—or by measurement at two selected wavelengths, when interfering colours are produced (galactose). Estimations in the presence of maltose or lower dextrins may be made after preliminary fermentation with brewer's yeast, though the slow and variable fermentation rates of the latter, presumably to be associated with differences in the degree of polymerization of the dextrins capable of giving a red colour, may present special difficulties. When cellobiose is present an approximation to the correct lactose concentration can be made by first acting upon the solution with emulsin, which hydrolyses β-glycosides much more rapidly than lactose.

SUMMARY

1. A method is described for the semi-micro estimation of lactose; modifications are given for its application to solutions containing lactose in the presence of glucose and glycogen, and in tissue extracts, and more general recommendations made for its use when other contaminating sugars are present.

2. The method is most sensitive for lactose concentrations in the range 0·05–0·2 %.

We wish to express our thanks to Prof. D. C. Harrison for his interest in the progress of this work and to Dr Q. H. Gibson for valuable suggestions with regard to the graphical presentation.

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Displacement Chromatography on Synthetic Ion-exchange Resins

4. THE ISOLATION OF GLUCOSAMINE AND HISTIDINE FROM A PROTEIN HYDROLYSATE

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In Part 3 of this group of papers (Partridge, 1949) the fractionation of the hydrochloric acid hydrolysis product of commercial egg albumin was described. A displacement chromatogram, using a column packed with 'Zeo-Karb 215' showed the presence of seven discrete bands each of which contained simple mixtures of amino-acids. These were identified by carrying out partition chromatograms on filter paper by the method of Consden, Gordon & Martin (1944). Band VI contained histidine together with an unknown basic substance. This substance has now been identified as glucosamine and both glucosamine and histidine have been isolated from the amino-acid mixture in a pure condition. The amount of glucosamine in the hydrolysis product is very small and its origin is probably the ovomucoid contained in white of egg. Histidine is also present in small amount, the content of this amino-acid in crystalline ovalbumin being about 2·4 % (Chibnall, 1945).

The following is the order in which the more basic amino-acids appear in the effluent from a column of 'Zeo-Karb 215' when ammonia solution is used as the displacement developer: leucine, histidine, glucosamine, lysine, ammonia. The affinity of glucosamine for the resin is not much greater than that of histidine, and separation is only partial with columns of normal length. It has been pointed out (Partridge & Westall, 1949; Davies, 1949) that when the displacement developer is a free base, the order of displacement reflects the ability of the stronger bases to control the pH of the aqueous phase and thus to depress the cationic form of the weaker bases.