alternative source for a starting material is imperative[3-6]. In this context, phytosterols, which are also used for the production of steroidal drugs, are of some importance. The already-known sources are not commercially viable because of poor recovery of sterols and an cumbersome process of extraction. In the present paper pressmud, which is a waste product of sugar factory, is proposed as an alternative source of phytosterols with a simple extraction method.

Experimental procedure and results. Phytosterols have been extracted from unfermented and fermented pressmud. For the former, a modified method[7] was followed. In the latter case phytosterols were extracted from the slurry obtained after fermentation of the pressmud with methanogenic bacteria at 31 ± 2 °C for 40 days. In this process extraction of phytosterols became easy because of biodegradation of lipophilic compounds by methanogenic bacteria.

To extract phytosterols from the residue obtained from the fermented pressmud, it was dried in an oven for 24 h at 100 °C to a moisture content of 2–3%, and was powdered and sieved (100 mesh) to remove bagasse.

One hundred grams of the powdered residue was then refluxed with a solvent mixture of benzene, petroleum ether and 2 N ethanolic KOH (10:5:1) for 12 h in a 5-liter Ca Soxhlet extraction flask. The extract so obtained, after decantation, was distilled in a 2-liter flask until 20 ml slurry was left. The slurry was transferred to a pre-weighed Petri-plate. After drying off the solvent at 80 °C in an oven, the slurry was cooled to room temperature. A soft cake weighing 8 g was thus obtained.

Extraction of phytosterols from the soft cake. For the selective solubilization of phytosterols, 8 g of the soft cake was refluxed with 80 ml of methylcyanide for 30 min. The hot extract was decanted into a pre-weighed Petri-plate and was allowed to cool at room temperature. The precipitate so obtained weighed 3.05 g and contained a mixture of phytosterols. This was again refluxed with 50 ml of isopropanol, and yielded 1.2 g of resin and 3.75 g of an undigested sticky slurry. The crude mixture of phytosterols separated by methylcyanide was purified on a column of neutral alumina using hexane, diethyl ether and methanol as solvents (table 1).

The percentage of individual sterols in the mixture was analyzed by GLC on a Gas Chromatograph Model Varian 3700 equipped with FID having a glass column packed with Chromosorb W(HP) 80–100 mesh and 3% OV-17 at column temperature of 275 °C. The chromatographic peaks were identified using authentic samples. The percentage composition of phytosterols obtained from the fermented and unfermented pressmud has been calculated (table 2).

Discussion. Pressmud is reported to contain 0.33% of phytosterols[8], but their low recovery and a cumbersome method of extraction has made its use uneconomical. However, its use after fermentation with methanogenic bacteria has made it a great potential source of phytosterols. During anaerobic fermentation of the pressmud carbohydrates including cellulose, proteins, and lipophilic compounds such as waxes were degraded into methane and CO_2. This resulted in approximately a 10-fold increase in the sterol content in the fermented samples over unfermented ones. The relative percentage of individual sterols in the mixture, however, remained unaltered. The GLC analysis of the purified mixture gave four peaks which were identified as those of brassicasterol, campesterol, stigmasterol and β-sitosterol. The amount of sterol was found to be 68.7% β-sitosterol, 18.4% stigmasterol, 12.9% of a mixture of campesterol and brassicasterol.

Pressmud has a distinct advantage over other sources of phytosterols in the country because of its cheap and plentiful availability (0.2 million tons per annum), besides being a potential raw material for the production of biogas.

Table 1. Amount of purified phytosterols obtained from pressmud by column chromatography using different solvents

Eluting agents	Compound eluted (g)	% of sterol
Hexane fraction	0.085	10.1
Diethyl ether fraction	3.469	87.6
Methanol fraction	0.078	3.0

Table 2. Percent composition of sterols obtained from unfermented and fermented pressmud

Mud	Individual sterol (% ± SD)		
	Brassicasterol and campesterol	Stigmasterol	β-Sitosterol
Unfermented pressmud	12.8 ± 0.04	18.3 ± 0.07	68.9 ± 1.90
Fermented pressmud	12.9 ± 0.06	18.4 ± 0.09	68.7 ± 1.85

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2 Goswami, P.C., Nag, B., Sharma, A.K., Borthakur, A., Singh, S.D., and Baruah, J.N., Curr. Sci. *52* (1983) 606.
3 Mathur, S.N., Srivastava, S.K., Srivastava, R.A.K., and Mall, D.K., Curr. Sci., communicated (1984).
4 Alo Sokar, L.V., and Chadha, Y.R. (ed.), Diosgenin and Other Steroidal Drug Precursors. Publication and Information Directorate, C.S.I.R., New Delhi, India (1979).
5 Fieser, L.F., and Fieser, M., Steroid. Reinhold Publication Corporation, New York 1959.
6 Chakravarti, R.N., Chakravarti, D., Mitra, M.M., Dasgupta, B., and Maiti, P.C.J., Sci. Ind. Res. *C15* (1956) 89.
7 Bose, S., and Gupta, K.C., 29th Proceedings of the Sugar Technologists Association, India (1961).
8 Weinberg, A., Dt. ZuckInd. *34* (1909) 629.

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Chromatofocusing coupled with automated assay for β-hexosaminidase isoenzymes in GM_2 gangliosidosis[1]

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Summary. Good separations of the two major β-hexosaminidase forms from human leukocytes were achieved by chromatofocusing, a technique which separates proteins on the basis of their isoelectric points. The use of an automated and reliable method is described for the identification of homozygotes and carriers of the GM_2 gangliosidosis.
Key words. Chromatofocusing; β-hexosaminidase; GM_2 gangliosidosis.

The lysosomal enzyme hexosaminidase (β-2-acetamido-2-deoxy-D-hexoside acetamidodeoxy-hexohydrolase, EC 3.2.1.51, Hex) is widely distributed in nature[3-5]. The isoenzymes A and B have been characterized from different tissues of normal

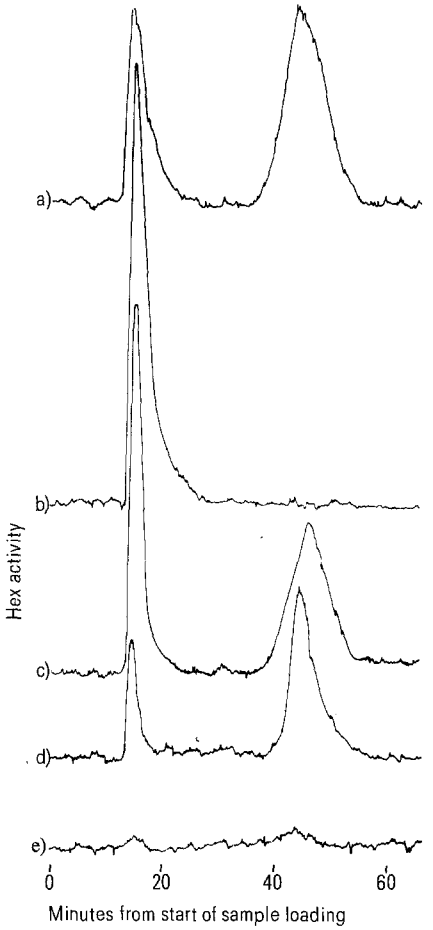
subjects and have been separated by DEAE-cellulose chromatography[6]. The isoenzymes levels show changes in GM_2 gangliosidosis, a group of lysosomal ganglioside storage diseases[7]. Almost complete deficiency of the isoenzyme A is responsible for the Tay-Sachs disease[8], while complete absence of the isoenzymes A and B is the cause of Sandhoff disease[9]. The carriers of the gene for Tay-Sachs disease can be detected by determining the Hex A activity in plasma and/or isolated peripheral leukocytes.

The isoenzyme A is more heat labile than B and it is usually determined as the difference between the total and the thermostable activities, calculated by manual or automated methods; with these methods carrier status could not be reliably determined on the basis of Hex A activity in a small but appreciable number of subjects[10].

The present report describes the use of chromatofocusing[11] coupled with automated enzyme assay for evaluating both A and B Hex isoenzymes.

Materials and methods. Chemicals. The following materials were obtained from the indicated sources: 4-methylumbelliferyl-N-acetyl-glucosaminide, 4-methylumbelliferone and human albumin from Sigma Chemical Co., St. Louis, MO, USA. Bovine gammaglobulin and Bio-Rad assay solution from Bio-Rad Laboratories, Richmond, CA, USA. Polybuffer exchanger PBE-94 and Polybuffer 74 for automated chromatofocusing from Pharmacia Fine Chemical Uppsala, Sweden. All other chemicals used were of analytical grade.

Specimens. The study was carried out on 10 control subjects, five patients and nine obligate heterozygotes for Tay-Sachs disease, then on two patients and four obligate heterozygotes for Sandhoff disease. Leukocytes were isolated from peripheral blood by the procedure described by Kampine et al.[12] and sonicated.



Resolution of Hex isoenzymes from leukocytes of controls (a), Tay-Sachs patients (b), Tay-Sachs carriers (c), Sandhoff carriers (d) and Sandhoff patients (e) by chromatofocusing on PBE-94 coupled with automated enzyme assay. The first peak is Hex B (basic), the second peak is Hex A (acid).

Enzyme assay. Hex activity was assayed at 4.5 essentially as described earlier[13]. One enzyme unit was defined as the amount of enzyme that converts 1 nmole of substrate/h into 4-methylumbelliferone at 37 °C. The protein content was determined by the procedure of Bradford[14] with bovine gammaglobulin as standard. Specific activity was expressed as units/mg protein.

Automated chromatofocusing on PBE-94. Leukocyte suspensions were analyzed at room temperture by an automated chromatofocusing on polybuffer exchanger PBE-94 using a Technicon autoanalyzer II (Technicon Instruments Ltd, England) to separate Hex isoenzymes. Column length and diameter, pH gradient, sample size, and flow rate were all varied to determine optimum conditions for automated chromatofocusing. The column effluent was mixed with a fluorogenic substrate by a peristaltic pump. After the incubation to promote the hydrolysis, we introduced alkali to stop the reaction and to enhance the fluorescence of the product. Hex activity was thus continously monitored and the isoenzyme profiles recorded directly on the chart. The method for carrying out chromatographic run was the following: 100 µl of sample diluted in 25 mM piperazine-HCl, pH 5.5, was loaded into a PBE-94 microcolumn (4 × 70 mm), equilibrated with the same buffer. 25 min after sample injection, 0.075 mmoles/pH unit/ml polybuffer 74 pH 4.0 was pumped through the column to make linear pH elution gradient. Column effluent (0.6 ml/min) was continuously mixed with buffered substrate (1 mM 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide in 25 mM citric acid/50 mM sodium phosphate pH 4.5, containing 0.1 mg/ml of human albumin at a flow rate of 0.32 ml/min). The mixture passed through a coil at 37 °C for 8 min and the reaction was stopped by 0.4 M glycine buffer pH 10.6 added at a rate of 0.8 ml/min. The intensity of fluorescence was measured and the isoenzyme profiles recorded.

Results and discussion. The total Hex activities (U/mg prot.) in leukocytes were: 930 ± 225 in normal subjects, 710 ± 210 in Tay-Sachs obligate heterozygotes, 765 ± 150 in Tay-Sachs patients, 380 ± 50 in Sandhoff carriers and practically absent in Sandhoff homozygotes. Total Hex determination does not allow the differentiation between controls and homozygotes and heterozygotes of Tay-Sachs disease, as already reported[10–13].

By chromatofocusing coupled with automated Hex assay it was possible not only to characterize qualitatively the Hex isoenzymes, but also to quantify them by measuring the peak areas and to calculate their respective percentages.

Chromatofocusing coupled with automated assay revealed two major Hex isoenzymes in normal leukocytes: B (basic form) and A (acid form). The B isoenzyme passed straight through the column equilibrated at pH 5.5 and it represented 32 ± 4% of the total Hex activity. The A isoenzyme was eluted only when the column pH shifted towards 4 by the gradient obtained using polybuffer 74, pH 4.0; its activity was 68 ± 6 of the total Hex activity (figure, A).

In Tay-Sachs patients the A form was undetectable, while the B isoenzyme was greatly increased compared to normal controls, representing almost 100% of the total Hex activity (figure, B). An increase of the B form was detected also in obligate Tay-Sachs heterozygotes; the A form was present, but its profile was reduced with respect to controls, and represented only 42 ± 5% of the total activity (fig., C).

In Sandhoff carriers two peaks were present, but smaller than those found in normal subjects. In particular the isoenzyme B was reduced, since it was only 22 ± 4 of the total activity instead of 32 ± 4 as found in normal leucocytes (fig., D). In Sandhoff patients the elution profile was flat showing the absence of both A and B isoenzymes (fig., E).

The results obtained agree with the data reported in the literature using other methods[13,15,16]. The absence of Hex A, responsible for Tay-Sachs disease, and the severe dificiency of both A and B isoenzymes in Sandhoff disease has been clearly demonstrated in our patients. The reduced percentage of Hex A, less than 45%, is the hall-mark of Tay-Sachs carriers[10]; in our obligate heterozygotes the A isoenzyme percentage agreed well with the above value. Lowden et al.[15] stated that the detection of Sandhoff carriers requires the concomitant presence of low Hex total activity and reduced Hex B percentage. Our findings in leukocytes of obligate Sandhoff heterozygotes support these criteria.

In conclusion, by chromatofocusing coupled with automated assay it has been possible to obtain good, rapid and reliable separations of leukocyte hexosaminidase isoenzymes. This technique can be usefully employed for the diagnosis of GM_2 gangliosidosis; the identification of Tay-Sachs and Sandhoff heterozygotes was also possible, but the utilization of the method for this purpose require further confirmation on a larger number of subjects.

In this study leukocytes were chosen, since they represent the best source of enzyme, and the differences in Hex activity and isoenzyme pattern between controls, heterozygotes and GM_2 patients are clearly evident. For the application of these techniques in a large screening program it would be important to establish whether or not more easily available sources, such as tears and serum, could be used.

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3 Li, S.C., and Li, Y.T., J. biol. Chem. *245* (1970) 5153.
4 Verporte, J.A., Biochemistry *13* (1974) 793.
5 Orlacchio, A., Maffei, C., Binaglia, L., and Porcellati, G., Biochem. J. *195* (1981) 383.
6 Robinson, D., and Stirling, J.L., Biochem. J. *107* (1968) 321.
7 O'Brien, J.S., Okada, S., Fillerup, D.L., Veath, M.L., Adornato, B., Brenner, P.H., and Leroy, J.G., Science *172* (1971) 61.
8 Okada, S., and O'Brien, J.S., Science *165* (1969) 698.
9 Sandhoff, K., FEBS Lett. *4* (1969) 351.
10 O'Brien, J.S., The gangliosidoses in the metabolic basis of inherited disease, 5th edn, vol.46, p.945. Eds J.B. Stanbury, J.B. Wyngaarden, D.S. Fredrickson and J.L. Goldstein. McGraw-Hill, New York 1983.
11 Sluytherman, L.A., and Elgersman, O., J. Chromat. *150* (1978) 17.
12 Kampine, J., Brady, R.O., Kanfer, J.N., Feld, M., and Shapiro, A., Science *155* (1967) 86.
13 Suzuki, Y., Berman, P.M., and Suzuki, K., J. Pediat. *78* (1971) 643.
14 Bradford, M.M., Ann. Biochem. *72* (1976) 248.
15 Lowden, J.A., Ives, E.J., Keene, D.L., Burton, A.L., Skomorowski, M.A., and Howard, F., Am. J. human Genet. *30* (1978) 38.
16 Toma, S., Coppa, G.V., Donnelly, P.V., Ricci, R., and Di Ferrante, N., Carbohydrate Res. *96* (1971) 271.

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An improved flow-through chamber for time-lapse film analysis of oogenesis and embryogenesis[1]

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Summary. A simple flow-through chamber for time-lapse film analysis of developing organisms has been constructed. The medium is replaced efficiently and uniformly in the chamber which is, therefore, ideally suited for studying the effect of various drugs on development.
Key words. Time-lapse film; flow-through chamber; oogenesis; embryogenesis; insects.

Time-lapse film analysis has been used as a tool to study embryogenesis and oogenesis in a number of insects including *Drosophila*[2,3]. For long-term film studies the design of the flow-through chamber is of importance if cultivation artifacts are to be avoided. For example, when closing the chamber, hydrostatic pressure may build up due to capillary forces in the inlet and outlet connections, and during the experiment uneven flow in the chamber or insufficient replacement of culture medium may lead to local oxygen depletion or local heating of the medium by the microscope lamp. The design of our film chamber minimizes these potential artifacts. We are particularly interested in being able to replace the culture medium fast and efficiently by a test solution (for example culture medium containing various inhibitors) whose effect on normal development can then be studied in time-lapse films. The design of the chamber is illustrated in figures 1 and 2. The two hatched parts are made of plexiglass. If sterile conditions are essential, the chamber can be washed in 0.1% diethyl pyrocarbonate[4] or exposed to UV, and the medium is pumped through a sterilizing micropore filter before entering the chamber.

The main advantages of the chamber are:

1. The distance between slide and coverslip can be varied by screwing the lid until the desired distance is obtained and the object is immobilized between slide and coverslip.
2. Trapped air bubbles anywhere in the chamber can be removed by a vent. By turning the lid the vent opening in the chamber is moved to the position of the bubble, the vent is opened and the air bubble removed.
3. When closing the chamber lid little pressure builds up inside the chamber. Multiple inlet and outlet openings (fig.3a: position of inlet openings indicated by colored medium) give a large cross-sectional area and hence capillary forces are minimized.
4. Due to the large number of inlet and outlet openings the culture medium streams fast and rather evenly through the chamber. Replacement of water with trypan blue-colored water in the chamber illustrates this point (fig.3a–d). If the object to be studied is placed in a central position between inlet and outlet openings any drugs to be tested reach the object within 60 s using a moderate flow-rate of 0.37 ml/min (fig.3). Small concentration differences in the chamber equilibrate within a few minutes.

The chamber has only two movable parts and is easy to use. When closing the chamber displacement of eggs or follicles can be prevented by coating the slide with polylysine. To obtain a constant flow-rate a motor-driven 50 ml syringe (Unita I, Braun Melsungen) has been used rather than a peristaltic pump. The film chamber described above has been employed successfully in analyzing the effects of various drugs on cytoplasmic streaming in vitellogenic follicles of *Drosophila*.