Comparison of incorporation of UDP-galactose-^{3}H in supernatant and pellet fractions and the effect of different concentrations of crude extract

	Protein (mg)	CPM
Assay mixture without crude extract	0	249
Assay mixture without UDP-galactose-^{3}H with supernatant II	4.5	99
Complete assay mixture with supernatant II	0.9	5426
	1.8	45025
	4.5	48483
	9.0	70850
Complete assay mixture with pellet	1.16	5502
	2.32	14430
	5.8	26900
	11.6	35764

Incubation was carried out as described in the methods. Each assay was brought to a constant volume with 0.025 M glycylglycine buffer pH 7.5.

was subjected to hydrolysis in 3N HCl at 100 °C for 2 h. The HCl was evaporated and the residue dissolved in 0.1 ml of H_2O. Descending chromatography was carried out on Whatman 1 paper in ethyl acetate-pyridine-water (8:2:1 v/v) for 19 h at 25 °C. The chromatogram was developed with diphenylamine-aniline[10] and 1-cm wide strips were cut into squares and counted in a Beckman LS-230 liquid scintillation counter with toluene-phosphor (Fisher). The following standard monosaccharides were used for chromatography: D-glucose, D-galactose, n-acetylglucosamine and n-acetylgalactosamine.

Results. These studies provide evidence for the presence of an enzyme in Neurospora crassa that is able to catalyze the transfer to galactose from UDP-galactose to a galactose acceptor. This enzyme will be referred to as galactosyltransferase. Incorporation of UDP-galactose-^{3}H was greater in the supernatant II than in the pellet as shown in the table. Incorporation was shown to increase in a linear manner when the amount of protein added to the assay mixture was increased. These data are presented in the table. The specific activity of the enzyme in the supernatant II fraction increased linearly with a maximum of 25,014 cpm/mg protein. The maximum activity shown in the pellet fraction was 6220 cpm/mg. Thus there was a 4fold difference in the specific activity of the 2 fractions. The enzyme showed an absolute requirement for manganese for its activity. The radioactive carbohydrate incorporated was identified by descending chromatography. All radioactivity was shown to cochromatograph with galactose in the supernatant II fraction only.

Discussion. The restricted growth exhibited by wild type Neurospora when grown in a culture medium containing galactose as the sole carbon source suggests the importance of galactose in the morphogenesis of Neurospora[7]. A possible regulatory role for galactose polymers in Neurospora morphogenesis has been previously shown[6]. However, the biochemical nature of the effect of galactose has not yet been elucidated.

The present investigations describe a galactosyltransferase that is able to effect the formation of galactose polymers in Neurospora. Further studies would be of interest in determining the precise role of galactose and galactosyltransferases in the growth and morphology of Neurospora. One approach to such future investigations would involve the ability of galactosyltransferase to confer concanavalin A agglutinability to certain cell types[11]. Concanavalin A, a lectin obtained from Jack beans, is known to agglutinate slime mutant of Neurospora[12]. This observation would offer the opportunity for a biochemical genetic analysis of galactosyltransferase by selecting for mutants of this enzyme due to their inability to agglutinate in the presence of concanavalin A.

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2 N. C. Mishra, in: Advances in Genetics, Vol. 19. Ed. E. W. Caspari. Academic Press, New York 1977.
3 E. L. Tatum, Naturw. Rdsch. Stuttg. *26*, 140 (1973).
4 T. M. Harold, Biochem. biophys. Acta *57*, 59 (1972).
5 P. R. Mahadevan and E. L. Tatum, J. Bact. *90*, 1073 (1965).
6 J. L. Reissig and J. E. Glasgow, J. Bact. *106*, 882 (1971).
7 J. B. Rand, Ph. D. thesis, The Rockefeller University, New York 1975.
8 H. J. Vogel, Am. Nat. *98*, 435 (1964).
9 O. H. Lowry, N. H. Rosebrough, A. L. Farr and R. J. Randall, J. biol. Chem. *193*, 265 (1951).
10 I. Smith. Chromatographic and Electrophoretic Techniques, vol. 1, p. 246. Ed. Interscience Publishers, Inc., New York 1960.
11 D. K. Podolsky, M. M. Weiser, J. T. LaMont and K. J. Isselbacher, Proc. nat. Acad. Sci. US *71*, 904 (1974).
12 H. F. Terenzi, M. M. Flawia and H. N. Torres, Neurospora News Letter *21*, 19 (1976).

(Ca-Mg)ATPase activity of human erythrocyte membranes: Influence of incubation buffer[1]

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Summary. Activity of the (Ca-Mg)ATPase of human red blood cell membranes is highly dependent on the specific buffer used in the ATPase assay. Activity is highest in histidine and/or imidazole buffers and is lowest in HEPES buffer.

There is now general agreement that the (Ca-Mg)ATPase of the human erythrocyte (RBC) membrane is associated with the active transport of Ca from the cell[2]. There is less agreement, however, on the magnitude of the (Ca-Mg)-ATPase activity of seemingly comparable preparations

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2 H. J. Schatzmann, Curr. Top. Membrane Transp. *6*, 126 (1975).

in various laboratories[3–6]. We wondered whether differing ATPase assay conditions, in particular, buffer composition, could account for some of the observed differences. This has occurred in other systems[7,8]. To investigate this, 10 different buffers or buffer combinations were examined for their effects on (Ca-Mg)ATPase activity when used in an otherwise defined ATPase incubation medium.

Materials and methods. All chemicals were purchased from Sigma. Membranes were prepared[9] from RBC hemolyzed in 310 imOsm imidazole buffer (pH 7.4), washed 4 times in 20 imOsm imidazole buffer (pH 7.4), and washed once in 40 mM-40 mM histidine-imidazole buffer (pH 7.1). An equal volume of the latter buffer was added to the last pellet of packed membranes. The resulting preparation (final protein content was about 10 mg/ml suspension) was stored on ice and assayed the following day. All steps of preparation took place at 0–5 °C. Protein content of the suspension was determined by the Lowry method[10] using bovine serum albumin as a standard. The ATPase incubation medium for Mg-ATPase contained (in a total volume of 2 ml): 0.9 ml buffer (pH 7.1), 3 mM ATP (Na_2 ATP-neutralized to pH 7.1), 3 mM $MgCl_2$, 80 mM NaCl, 15 mM KCl and 0.1 mM ouabain. $CaCl_2$ (0.1 mM) was included in addition in the tubes for the determination of (Ca-Mg)ATPase. (Ca-Mg)ATPase was taken as the ouabain-insensitive, Ca-stimulated, Mg-dependent ATPase minus ouabain-insensitive, Mg-stimulated ATPase. Activity is expressed as μmole P_i/mg protein/h. The tubes for Mg-ATPase also served as blanks to correct for the nonenzymatic hydrolysis of ATP and for any inorganic phosphate content of the membrane suspension. All tubes were incubated in duplicate at 37 °C for 1 h. The reaction was initiated by addition of substrate and terminated by addition of 1 ml of ice-cold 1.5 N perchloric acid. After mixing and centrifugation, 1 ml of supernate was assayed for inorganic phosphate[11].

ATPase assays were performed with the addition of 0.9 ml of 10 different buffers (pH 7.1) in the 2 ml incubation medium: 224 mM histidine, 40 mM-40 mM histidine-imidazole, 95 mM-95 mM histidine-imidazole, 165 mM imidazole, 125 mM Tris [tris(hydroxymethyl) aminomethane], 174 mM ACES [N-(2-acetamido)-2-aminoethanesulfonic acid], 158 mM BES [N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid], 158 mM MOPS [3-(N-morpholino)propanesulfonic acid], 141 mM TES [N-tris(hydroxymethyl)methylaminoethanesulfonic acid], and 139 mM HEPES (N-2-hydroxyethylpiperazine-N′-ethanesulfonic acid). Because of differing pK_a's[12] the concentration of each buffer varied in order to yield a total osmolarity for the incubation medium of 305 ± 5 mOsm (mean ± SE, n = 9). 1 exception was histidine-imidazole (40 mM-40 mM), which gave a total osmolarity for the incubation medium of 266 mOsm. This buffer was included because it is the standard buffer used for most ATPase assays in our laboratory.

Results and discussion. The results (table) show that (Ca-Mg)ATPase activity of RBC membranes is highly dependent on the specific buffer used. Greatest activity was obtained with 40 mM-40 mM histidine-imidazole and 224 mM histidine buffers (95 mM-95 mM histidine-imidazole buffer gave approximately the same result). All other buffers produced significantly less (Ca-Mg)-ATPase activity. It is worth noting that: a) (Ca-Mg)-ATPase activity did not depend on the pK_a of the buffer ($r = 0.56$). b) BES, MOPS, HEPES, ACES and TES are compounds with pK_a's ranging from 6.9 to 7.55. These buffers should be advantageous for use at physiological pH's. However, all of these yielded significantly less (Ca-Mg)ATPase activity when used as the buffer in the ATPase assay incubation medium. c) Tris has a pK_a of 8.3 and thus, is an inappropriate buffer at pH's of less than 7.3 (even though often used at those pH's). This, too, produced decreased (Ca-Mg)ATPase activity. However, Tris inhibited (Ca-Mg)ATPase activity no more than other buffers (e.g., TES, ACES and HEPES) that would be good buffers at pH 7.1.

The mechanism of the buffer effect is not clear. The data demonstrate significant differences based on buffer composition at a given pH. The mechanism is not osmolarity differences since this factor was kept constant and probably not ionic strength effects because there was no correlation between the pK_a of a given buffer and the resulting (Ca-Mg)ATPase activity. This work suggests that buffer effects may account for some apparent differences in (Ca-Mg)ATPase activities reported from various laboratories.

Influence of different buffers on (Ca-Mg)ATPase activity

Incubation buffer added	pK_a	n	(Ca-Mg)ATPase activity	Maximal activity (%)
224 mM histidine	6.05	9	3.42 ± 0.23 n.s.	100 ± 7
40 mM-40 mM histidine-imidazole	(6.55)	9	3.41 ± 0.24	100 ± 7
95 mM-95 mM histidine-imidazole	(6.55)	3	3.23 ± 0.48 n.s.	94 ± 14
165 mM imidazole	7.05	9	2.86 ± 0.19*	84 ± 6
158 mM BES	7.15	4	2.77 ± 0.24*	81 ± 7
158 mM MOPS	7.15	4	2.53 ± 0.26*	74 ± 8
141 mM TES	7.5	4	2.27 ± 0.21*	66 ± 6
174 mM ACES	6.9	9	2.26 ± 0.16*	66 ± 5
125 mM Tris	8.3	10	2.24 ± 0.11*	65 ± 3
139 mM HEPES	7.55	4	2.10 ± 0.16*	61 ± 5

(Ca-Mg)ATPase activity (μmole P_i/mg protein/h, mean ± SE) was determined for RBC membranes incubated in different buffer systems. All data were compared to that of 40 mM-40 mM histidine-imidazole buffer by Student's t-test. pK_a s in parentheses are calculated pK_a's. *Significantly less than 40 mM-40 mM histidine-imidazole buffer, $p < 0.0005$.

3 S. A. Feig and G. Guidotti, Biochem. biophys. Res. Commun. *58*, 487 (1974).
4 E. L. Watson and K. G. Tolman, Res. Commun. chem. path. Pharmac. *6*, 1077 (1973).
5 G. H. Bond and J. W. Green, Biochim. biophys. Acta *241*, 393 (1971).
6 H. J. Schatzmann and G. L. Rossi, Biochim. biophys. Acta *241*, 379 (1971).
7 R. W. Butcher and E. W. Sutherland, J. biol. Chem. *237*, 1244 (1962).
8 P. E. Dresel, Can. J. Physiol. Pharmac. *52*, 1025 (1974).
9 F. F. Vincenzi and M. L. Farrance, Pharmacologist *17*, Abst., 199 (1975).
10 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, J. biol. Chem. *193*, 265 (1951).
11 C. H. Fiske and Y. SubbaRow, J. biol. Chem. *66*, 375 (1925).
12 N. E. Good and S. Izawa, Meth. Enzym. *24B*, 53 (1972).