chloride; ν_{max}^{Nujol} 3360, 1625, 1605 cm^{-1}. The NMR of the compound showed it to be 5, 7-dihydroxy-6, 8-di-C-prenyl flavanone (VII).

Since a number of hydroxy chromenoflavanone are known to occur in Nature, it was considered worthwhile to prepare the two isomeric chromenes from II and I. Thus, when II was treated with DDQ in benzene solution for two hours the corresponding chromene (VIII) was obtained in 70% yield, as colourless needles from methanol, ($C_{20}H_{18}O_4$), m.p. 132–33°; brown colour with ferric chloride; λ_{max} 270, 292 nm; ν_{max}^{Nujol} 1650 cm^{-1}. NMR ($CDCl_3$): δ 12·46 (*s*, 1H, chelated –O*H*), 7·45 (*s*, 5H, C_6H_5), 6·54 (*d*, J = 11 Hz, 1H, Ar–C*H*–CH–C̆–O), 6 01 (*s*, 1H, C_6–*H*), 5·26–5·49 (*m*, C_2–*H* and Ar–CH = C*H*–C̆–O–), 2·73–3·03 (*m*, 2H, C_3–H_2), 1·44 (s, 6H, $> C\langle^{CH_3}_{CH_3}$).

A similar reaction with I yielded the chromene (IX) in 70% yield. It was crystallised as colourless needles from methanol, ($C_{20}H_{18}O_4$), m.p. 100–101°; brown colour with ferric chloride; λ_{max} 266, 292 nm; ν_{max}^{Nujol} 1650 cm^{-1} NMR ($CDCl_3$): δ 12·41 (*s*, 1H, chelated –O*H*), 7·45 (*s*, 5H, $-C_6H_5$), 6·64 (*d*, J = 11 Hz, Ar–C*H* = CH–C̆–O–), 6·00 (*s*, 1H, C_8–*H*), 5·25–5·61 (*m*, 2H, C_2–*H*) and Ar–CH = C*H*–C̆–O–), 2·80–3·11 (*m*, 2H, C_3–H_2), 1·44 (s, 6H, $> C\langle^{CH_3}_{CH_3}$).

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DECRYPTIFICATION OF NITRATE REDUCTASE ACTIVITY IN THE YEAST *CANDIDA UTILIS*

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ABSTRACT

A diverse variety of chemicals and drugs are known to affect the cell walls of bacteria and yeasts resulting in an enhanced access of the intracellular enzymes to the added substrates. The ability of organic solvents, antifungal drugs, detergents, and chelating compounds to decryptify NAD(P)H : nitrate oxidoreductase (EC 1.6.6.2) activity in *Candida utilis*, has been investigated. Of these, toluene, butanol, 2-phenylethanol and amphotericin B, in that order, are effective. The degree of decryptification is dependent on the concentration of the agent, and at 2·5% (v/v), toluene-ethanol elicited highest enzyme activity. These permeated cell preparations greatly aid in the regulatory studies of various yeast enzymes *in situ*.

INTRODUCTION

INVESTIGATIONS concerning regulatory phenomena, *viz.*, induction and repression of various enzymes such as β-galactosidase in *Escherichia coli* were carried out *in situ* using permeated bacterial cells[1-3], thus doing away with the laborious process of preparing cell-free extracts. The growing awareness in the recent past of the fallacies inherent in inferring the *in vivo* situation from *in vitro* observations further strengthened the surge of interest in the development of novel methods to decryptify various enzymes and assay the activities *in situ* in permeated cells of a variety of organisms[4-10].

The detailed studies on the regulation of yeast nitrate reductase (EC 1. 6. 6. 2) (NAR) under investigation in our laboratory which involve determination of rapidly changing enzyme levels[8], necessitated the development of an alternate assay method, which is simpler, rapid and more sensitive than the conventional *in vitro* procedure.

The paper is concerned with the examination of the relative efficacy of different compounds in decryptifying NAR activity in induced *Candida utilis* cells. The typical method used to assay the decryptified NAR activity *in situ* using permeated *C. utilis* cells is also described.

MATERIALS AND METHODS

Chemicals.—Lab reagent grade benzene was purchased from Sarabhai M. Chemicals, and solvent ether from Alembic Chemical Works Co. Ltd., Baroda. Analytical reagent grade chloroform, DMSO, DMF, acetone, propanol, butanol, amyl alcohol, potassium nitrate, sulfur-free grade lab reagent toluene and polyethylene glycol 400 were purchased from BDH Ltd., Bombay, India. 2-Phenylethanol, amphotericin B, nystatin, griseofulvin, sodium salt of EDTA, NADH, FAD, DTT, and crystalline bovine serum albumin were obtained from Sigma Chemical Co, St. Louis, U.S.A. Miconazole (1-{2-(2, 4-dichlorophenyl)-2-[(2, 4-dichlorophenyl) methoxy]ethyl}-1 H-imidazole mononitrate) was a kind gift from

Janssen Pharmaceutica, B-2340, Beerse, Belgium, through the courtesy of Ethnor, Ltd., Bombay. Sulfanilamide was purchased from the Indian Drugs and Pharmaceuticals, Ltd., India and N-(1-naphthyl) ethylenediamine. 2 HCl from BDH, Ltd., Poole, England. Other chemicals were of analytical grade from BDH Ltd., Bombay, India or Riedel–De Haenag Seelze–Hannover, Germany.

Organism and Maintenance.—*Candida utilis* CBS 4511 was obtained from the Centraalbureau voor Schimmelcultures, Delft, The Netherlands. The yeast was maintained by subculturing once in two months on Sabouraud's glucose agar slants containing neopeptone, 1% ; glucose, 1% and yeast extract, 0·2%.

Media and Cultivation Techniques—The basal medium was essentially the same as described by Wickerham[11] with a few modifications, and contained potassium nitrate, 50 mM (N) as nitrogen source (induction medium). The yeast was cultured by inoculating liquid medium in 500 ml-Erlenmeyer flasks with 5% (v/v) of ~ 24 h culture, aerated by shaking on an Emenvee gyratory shaker (180 rpm) at 30° C. The cells in late exponential phase of growth ($\simeq$ 12 h after inoculation) were harvested, washed with chilled phosphate buffer, and packed by centrifugation at 6,000 × g for 5 min. at 4° C in a Sorvall RC 2–B centrifuge. The packed cells were stored frozen at — 10° C till use.

Induction of NAR.—The cells of *C. utilis* grown on KNO_3 (1%, w/v) as nitrogen source had high levels of NAR activity. The inducibility of NAR in *C. utilis* by NO_3^- was reported[12].

Preparation of Permeated Yeast Cells.—The general procedure of permeating the yeast cells was as follows : An aliquot of 0·05 ml chilled decryptifying agent was added to 2 ml of the cell suspension [100 mg (fresh wt) of induced cells/ml of 'preparation buffer', 0·1 M phosphate buffer, pH 7·0 containing 10^{-4} M DTT and $1·5 \times 10^{-4}$ M EDTA] in a 16 mm test-tube, and shaken for 2 min at 4° C on a Jay Vortex Mixer. Suitable aliquots of this preparation were used to assay NAR *in situ*.

Enzyme Assay :

NAR in situ.—The reaction mixture contained, KNO_3, 0·02 M ; 0·5 ml (50 mg fresh wt) of permeated cell preparation, and 0·1 M pyrophosphate buffer, pH 7·0 to give a final volume of 1 ml. The reaction was carried out 10 min. at 37° C and terminated by the addition of 1 ml of 1% sulfanilamide in 3 N HCl. The nitrite content of an aliquot of the supernatant obtained after low speed centrifugation was determined. There was no significant reduction of nitrite in blanks under the assay conditions.

Analytical Assay :

Nitrite.—The nitrite content of 1 ml-reaction mixture was determined colorimetrically by a modification of the diazo coupling procedure described by Snell and Snell[13], with 1 ml of 1% (w/v) sulfanilamide in 3 N HCl followed by 1 ml of 0·02% (w/v) N-(1-naphthyl) ethylenediamine dihydrochloride, and distilled water to give a final volume of 5 ml. After 10 min, the color intensity was determined at 540 nm with a Beckman model DU spectrophotometer. The absorbancy values were converted into nmoles of nitrite by multiplication with the slope of a nitrite concentration reference curve made with sodium nitrite.

Units and Specific Activity.—One unit of activity is defined as equivalent to that amount of the enzyme which catalyzes the formation of 1 nmole of nitrite per 1 min and the specific activity is expressed as units per mg of total extractable protein (55 mg/g fresh yeast).

Results

Relative Efficacy of Various Permeating Agents in Decryptifying NAR Activity in C. utilis.—To select a suitable permeating agent that can effectively decryptify NAR in *C. utilis*, cells from exponential phase of growth were harvested, washed, and suspended in the preparation buffer to give 100 mg (wet wt) of cells/ml, and treated with various permeating agents reported in the literature, such as different organic solvents including alcohols, membrane-affecting drugs, chelating agents, etc., according to the typical procedure, described under Materials and Methods. A comparison of the relative levels of NAR activity of the cells permeated by different solvents showed toluene to be the most effective, and benzene the next best (Table I). Acetone, DMSO, chloroform, ether and DMF were not efficient as permeating agents.

Butanol, followed by ethanol, was the best among the aliphatic alcohols tested in decryptifying NAR activity. Phenylethanol, the only aromatic alcohol tested was almost as effective as butanol. Although polyethylene glycol 400 and propanol did show some effect, the relative levels of NAR activity decryptified, however, were not significant.

A number of antifungal drugs, known to affect cell membranes of the susceptible organisms, were also tested for their efficacy in permeating yeast cells. Amphotericin B was most effective folowed by miconazole, a synthetic compound recently coming into increasingly wide use as a broad-spectrum antimicrobial drug.

TABLE I

Decryptification of nitrate reductase activity in C. utilis *by various compounds*

Solvent[a]	NAR[c] specific activity
Organic solvents:	
Toluene	18·0
Benzene	9·9
Acetone	1·1
DMSO	0·1
Alcohols:	
Butan-1-ol	16·2
2-phenylethanol	14·7
Ethanol	13·2
Polyethylene glycol 400	7·8
Propan-2-ol	5·4
Membrane-active drugs[b]:	
Amphotericin B	12·0
Miconazole	8·0

a used at a concentration of 5% (V/V).
b The final concentration was 1 mM
c Assayed *in situ* at 37°C for 10 min.

Nystatin and griseofulvin, at the concentrations tested, had little effect. Various detergents like Triton X-100, Brij, sodium deoxycholate either failed to bring about any decryptification of NAR activity or inactivated the enzyme. Similarly, EDTA[3] and bovine serum albumin[7] used by earlier workers as permeating agents, were not useful in decryptifying NAR activity of *C. utilis* cells.

Effect of Concentration of the Permeating Agent on Decryptification of NAR Activity.—Further, the effect of varying the concentration and composition of the permeating agent on decryptification of NAR activity was investigated. Figure 1 shows the effect of different concentrations of toluene, ethanol and their mixture on permeation of the yeast cells. Although toluene was very effective in permeating the yeast cells, its effect was enhanced significantly, when it was used in combination with ethanol, as indicated by increased levels of NAR activity. Maximal enzyme activity was elicited by 2·5% (v/v) toluene-ethanol (1 : 4, v/v). Higher concentrations of toluene-ethanol, however, were deleterious to the enzyme activity[14]. This could be due to the probable sensitivity of the enzyme to alcohol because toluene, by itself did not affect NAR activity at higher concentrations. Untreated cells, however, showed no evidence of decryptified NAR activity.

Discussion

Of various permeating agents examined, toluene, in combination with alcohol, was most effective in decryptifying NAR activity in *C. utilis* cells. A variety of substances including toluene[1-3,16,17] benzene[6], DMSO[8], polyene antibiotics[4], basic proteins[7], and chelating compounds[5], was reported to permeate bacterial and yeast cells. Toluene found extensive use in such studies for its selective action on the cells rendering them freely permeable to a wide variety of low molecular weight substances[16,17]. Our results substantiate this observation regarding the high retention of the enzymatic potential of the treated cells. The absence of NAR activity in cells treated with EDTA (widely used by other workers to permeate cells) may be the result of the inhibition by EDTA of the activity of NAR, a metalloflavo-protein rather than its failure to permeate yeast cells[14].

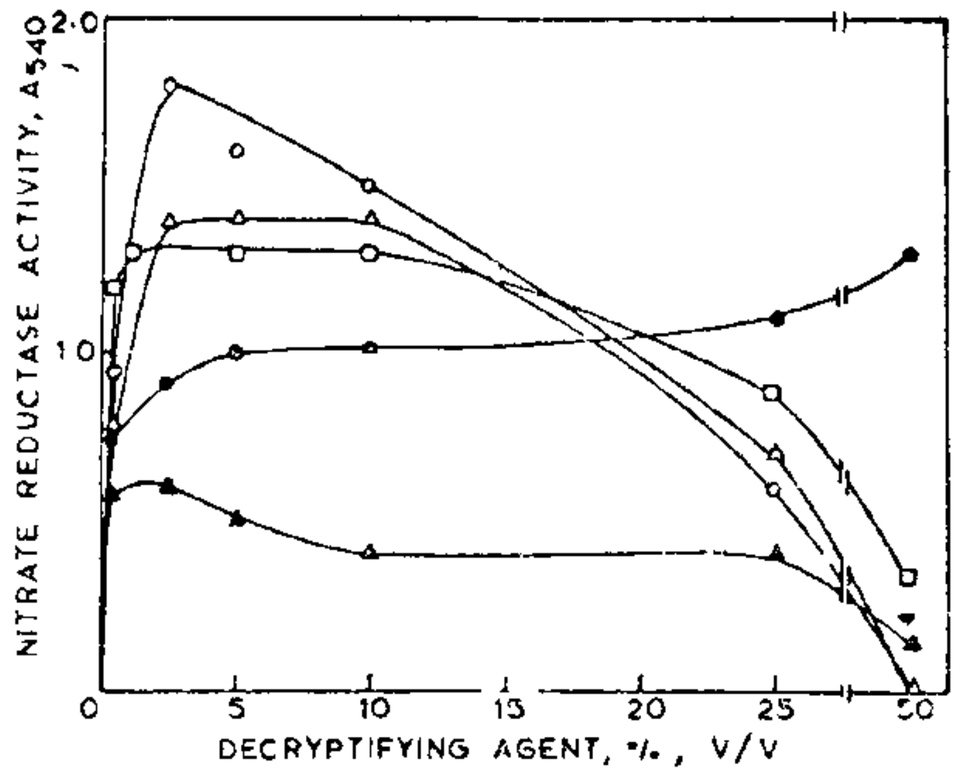

FIG. 1. Decryptification of nitrate reductase activity in *C. utilis* as a function of the concentration of the permeating agent. An O.D. of 1·0 at 540 nm corresponds to 10 nmoles of nitrite formed/min/mg protein. (●) toluene; (▲) ethanol; (□) toluene-ethanol. 1 : 1, v/v; (△) toluene-ethanol, 1 : 2, v/v; (○) toluene-ethanol, 1 : 4, v/v.

The toluene-treated cells showed high NAR activity under *in situ* conditions without added cofactors[19], contrary to the property of NAR *in vitro*, where the enzyme showed an absolute requirement for NAD (P) H[14,15]. However, the addition of coenzymes increased the *in situ* activity by 50%. The initial high *in situ* activity could be due to the participatiion of endogenous NAD (P) H and FAD in the reaction. Presence of adequate intracellular concentrations of NADH and cAMP in yeast cells was recently reported by Gancedo and Gancedo[18].

The proposed *in situ* assay, thus, is much simpler, less expensive, quite sensitive and reliable,

and enables one to overcome the apparent shortcomings attributed to the *in vitro* method of assay. The additional advantage that the cells need no other supplement of the coenzymes for routine assay of the enzyme activity *in situ*, makes this procedure uniquely distinct even over the other *in situ* assay methods known, where the only advantage over the conventional *in vitro* methods is that whole cells can be used in place of cell-free preparations. All our subsequent studies on the regulatory phenomena controlling the biosynthesis of NAR in *C. utilis*, which involve handling of innumerable concentrations, and assay of NAR activities at brief time periods, are conducted using the *in situ* technique and the results appear elsewhere.

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A RAPID SPECTROPHOTOMETRIC METHOD FOR THE QUANTITATIVE DETERMINATION OF DIOSGENIN IN *DIOSCOREA* TUBERS

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ABSTRACT

A rapid method for the assay of diosgenin in *Dioscorea* tubers has been developed Simultaneous hydrolysis and extraction of the tubers (50 mg) by 3 N HCl and hexane is carried out in 1½ to 2 hours. $SbCl_3$ (24% in $HClO_4$) has been used for developing colour The red colour formed is stable for 2 hours and is evaluated spectrophotometrically at 486 nm. One analyst can assay 8 to 10 samples per day by this method.

INTRODUCTION

DIOSGENIN is used in the synthesis of steroidal drugs. Several analytical procedures are available for the estimation of diosgenin in pure state or in *Dioscorea* tubers. Gravimetric procedure[1], gas liquid chromatography[2], colorimetry[3], densitometric thin-layer chromatography[4] and infra-red spectrometry[5] have been used. These methods involve more time, labour and material. The present paper describes a rapid method combining hydrolysis and extraction of *Dioscorea* tubers with spectrophotometric method for the determination of diosgenin in the extracts. The distinguishing features of this method are (*a*) small quantities (50 mg) of the dried plant material required, (*b*) hydrolysis and extraction achieved in 1½ to 2 hours, (*c*) avoiding additional solvents[3-6] and (*d*) use of smaller quantities of antimony ion for the colour reaction.

EXPERIMENTAL

Pure diosgenin was isolated from *Dioscorea deltoidea* and purified by preparative T.L.C. using $SbCl_3$ solution. (24% in 70% $HClO_4$) for detection.

Procedure

(*i*) *Preparation of Standard Curve.*—25 mg of pure diosgenin were dissolved in 250 ml hexane (Solution A) and 0·75 ml and 1·0 ml of this solution were taken in two tubes and the solvent removed by bubbling air. $HClO_4$ (5 ml) followed by 0·1 ml of $SbCl_3$ solution. (24% in 70% $HClO_4$)

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