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FURTHER PROPERTIES OF THE POLYAMINE OXIDASE OF BARLEY LEAVES

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Key Word Index—*Hordeum vulgare*; Gramineae; barley; *Pisum sativum*; Leguminosae; pea; polyamine; diamine; oxidases; *putrescine*; *spermidine*; *spermine*; *methylglyoxal-bis*(guanylhydrazone); *N-methyl-2-benzothiazolone* hydrazine.

Abstract—Polyamine analogues have been studied as potential inhibitors or substrates of barley leaf polyamine oxidase. $NH_2(CH_2)_3NH(CH_2)_{10}NH_2$ was particularly effective as an inhibitor of spermine oxidation at pH 4·5 ($K_i = 5 \times 10^{-6}$ M). Methylglyoxal-*bis*(guanylhydrazone) inhibited spermine oxidation only slightly ($K_i = 10^{-4}$ M). Activity with the polyamine analogues as substrates was generally 10% or less of the activity with spermine. The K_m for oxygen was 3×10^{-4} M. The K_m for spermine oxidation was independent of oxygen concentration. Using the *N*-methyl-2-benzothiazolone hydrazine reagent, 1-(3-aminopropyl)pyrroline was shown to be formed stoichiometrically by the enzyme on oxidation of spermine. The enzyme will not function as a dehydrogenase in the presence of oxygen with either potassium ferricyanide or dichlorophenolindophenol as electron acceptors. Activity in the leaves increased with age, up to 4 weeks. In the leaves of 11-week-old plants activity was lower than in leaves of 1-week-old plants. The enzyme was mainly associated with an easily-sedimented particulate fraction, and relatively small proportions were found in the cell wall or soluble fractions.

INTRODUCTION

ENZYMES capable of oxidizing polyamines occur in widely separated groups of animals, plants and micro-organisms.[1] The polyamine oxidase characterized in barley and maize (E.C. 1.5.3.3), and probably occurring in other members of the Gramineae, attacks the *secondary* amino groups of the naturally-occurring polyamines spermidine and spermine, to give 1-pyrroline and 1-(3-aminopropyl)pyrroline respectively.[2] With both substrates, 1,3-diaminopropane is also formed. This enzyme differs in many of its characteristics from the pea seedling amine oxidase (E.C. 1.4.3.6) which attacks the primary amino groups of a wide range of amines, especially the diamines putrescine and cadaverine, but also including spermidine and spermine; indeed, the barley leaf polyamine oxidase has greater affinities with enzymes described from the bacteria *Neisseria perflava* and *Hemophilus parainfluenzae* which oxidize spermidine and spermine,[3] and with an enzyme in *Micrococcus rubens* which is very active with putrescine as substrate but which will also attack cadaverine and spermidine.[4] A polyamine dehydrogenase from *Serratia marcescens* splits spermidine and possibly spermine at the same bond which is attacked by the barley leaf enzyme.[5] A further

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[1] KAPELLER-ADLER, R. (1970) *Amine Oxidases and Methods For Their Study*. Wiley-Interscience, New York.
[2] SMITH, T. A. (1974) *Phytochemistry* **13**, 1075.
[3] WEAVER, R. H. and HERBST, E. J. (1958) *J. Biol. Chem.* **231**, 647.
[4] ADACHI, O., YAMADA, H. and OGATA, K. (1966) *Agr. Biol. Chem.* (Tokyo), **30**, 1202; DESA, R. J. and BRANTNER, R. (1972) *J. Biol. Chem.* **247**, 5527.
[5] TABOR, C. W. and KELLOGG, P. D. (1970) *J. Biol. Chem.* **245**, 5424.

similarity between the polyamine oxidases of the Gramineae and those at least of *Micrococcus* and *Serratia*, may be found in the apparent presence of flavin adenine dinucleotide as a cofactor in both of these microbial enzymes,[5,6] and also in the polyamine oxidase found in maize,[7] a plant closely related to barley.

Since enzymes which split polyamines are the only known source of 1,3-diaminopropane, the presence of this diamine in the urine of man[8,9] and in bovine brain [10] suggests the operation of similar enzymes in higher animals. Further properties of the polyamine oxidase from barley leaves have now been studied and compared with those of the diamine oxidase from pea seedling cotyledons.

RESULTS AND DISCUSSION

Effect of polyamine analogues as inhibitors or substrates of barley leaf polyamine oxidase

Analogues of spermidine and spermine, in which the length of the tetramethylene chain was shortened or lengthened, caused competitive inhibition of spermine oxidation (Table 1). The K_i for the spermidine series decreased progressively with chain length from $x = 2$ to $x = 10$ (where x is the number of methylenes substituted for the tetramethylene chain). For the spermine series, K_i decreased from $x = 2$ to $x = 6$, and with $x = 6$, 8 and 10 the K_is were similar. The spermidine analogues $x = 8$ and $x = 10$ were particularly effective inhibitors ($K_i = 10^{-5}$ and 5×10^{-6} M respectively). This is probably related to the chain length, which is similar to that of spermine. Inhibition of spermine oxidation by the fully *N*-methylated spermidine was high ($K_i = 5 \times 10^{-5}$ M). The effect of all these analogues on spermidine oxidation was complicated by their ability to act as substrates for the enzyme at higher pH, and this interaction was not further investigated. In the present series of experiments, spermine at pH 4·5 was oxidized 40× faster than spermidine at pH 7·5. In previous work,[2] spermine at pH 4·5 was oxidized 14× faster than spermidine at pH 8. By contrast, the enzyme from maize leaves[7] oxidizes spermidine (optimum pH 6·3) at a rate which is 50% greater than the oxidation of spermine (optimum pH 5·5). At pH 7·5 activity of the barley leaf enzyme with diaminodipropylamine ($x = 3$) was slightly less than activity with spermidine, and activity with spermine was about 1% of that at pH 4·5. The latter value is somewhat greater than that previously found (0·2%),[2] possibly due to contamination of the spermine by spermidine in the present study. Similarly, values presented for oxidation of the spermine analogues at pH 7·5 may be over-estimated, since the analogues were contaminated with small quantities of the spermidine analogues which were difficult to eliminate. Activity of the enzyme at pH 4·5 was more selective than at pH 7·5. Compared with spermine (= 100%), the spermine analogue with the next highest activity ($x = 8$) had only 2·6% activity. At pH 4·5 all the spermidine analogues had a very low activity, spermidine itself was about 26× more active at pH 7·5 than at 4·5; previously this factor was found to be 35.[2]

The enzyme from *Neisseria perflava*[3] oxidizes spermidine and spermine by a similar mechanism to that demonstrated for the barley leaf enzyme. At pH 7, the optimum for

[6] YAMADA, H., ADACHI, O. and OGATA, K. (1965) *Agr. Biol. Chem.* (Tokyo), **29**, 1148.
[7] SUZUKI, Y. and HIRASAWA, E. (1973) *Phytochemistry* **12**, 2863.
[8] BREMER, S., KOHNE, E. and ENDRES, W. (1971) *Clin. Chim. Acta* **32**, 407.
[9] WALLE, T. (1973) in *Polyamines in Normal and Neoplastic Growth* pp. 355–365 (D. H. RUSSELL, ed.), Raven, New York.
[10] NAKAJIMA, T., WOLFGRAM, F. and CLARK, W. G. (1967) *J. Neurochem.* **14**, 1113.

spermine oxidation, the bacterial enzyme oxidizes spermine at about twice the rate of spermidine.[3] *Sym*-homospermidine, which occurs naturally in leaves of the sandalwood tree,[11] was oxidized by the barley leaf enzyme at 1% of the rate with spermine at pH 4·5, and at pH 7·5 the rate of oxidation was about 50% of the rate with spermidine; the K_i for spermine oxidation at pH 4·5 was greater than 5×10^{-3} M.

Methylglyoxal-bis(guanylhydrazone) (MGBG), which acts as a polyamine analogue in the inhibition of *S*-adenosylmethionine decarboxylase[12] had a K_i of 10^{-4} M for the inhibition of spermine oxidation by the barley leaf polyamine oxidase. The K_i for spermidine oxidation at pH 7·8 was 2×10^{-4} M. No evidence was found for the oxidation of MGBG at pH 4·5 or 7·8.

TABLE 1. EFFECT OF POLYAMINE ANALOGUES, METHYLATED AMINES AND DIAMINES AS INHIBITORS OR SUBSTRATES OF BARLEY LEAF POLYAMINE OXIDASE

	K_i (M) spermine as substrate (pH 4·5)	% Activity at 10^{-3} M (spermine at pH 4·5 = 100%) pH 4·5	pH 7·5
Spermidine analogues $NH_2(CH_2)_3NH(CH_2)_xNH_2$			
$x = 2$	5×10^{-3}	0·2	0·2
$x = 3$	2×10^{-4}	0·3	2·3
$x = 4$ (= Spermidine)	7×10^{-5}	0·1	2·6
$x = 6$	2×10^{-5}	<0·1	1·3
$x = 8$	1×10^{-5}	<0·1	1·7
$x = 10$	5×10^{-6}	0·3	1·7
Spermine analogues $NH_2(CH_2)_3NH(CH_2)_xNH(CH_2)_3NH_2$			
$x = 2$	5×10^{-4}	<0·1	1·2
$x = 3$	2×10^{-4}	<0·1	7·6
$x = 4$ (= Spermine)	—	100	1·2
$x = 6$	9×10^{-5}	0·4	6·4
$x = 8$	1×10^{-4}	2·6	11·1
$x = 10$	9×10^{-5}	1·3	1·7
N-Methylated amines			
$(Me)_2N(CH_2)_3N(Me)(CH_2)_4N(Me)(CH_2)_3N(Me)_2$	5×10^{-5}	<0·1	0·4
$(Me)_2N(CH_2)_3NH(CH_2)_3N(Me)_2$	4×10^{-4}	<0·1	0·6
$(Me)_2N(CH_2)_3NH_2$	1×10^{-3}	<0·1	0·5
Diamines			
Diaminopropane	$>5 \times 10^{-3}$†	<0·5	<0·5‡
Putrescine	4×10^{-3}*†	<0·5	<0·5‡
Cadaverine	$>5 \times 10^{-3}$†	<0·5	<0·5‡

* At pH 4·5 inhibition by putrescine appears to be partly non-competitive.

† With spermidine as substrate at pH 7·8 no inhibition could be detected.

‡ pH 7·8

The inhibition of spermine oxidation at pH 4·5 by the polyamine analogues and the effectiveness of the analogues as substrates at pH 4·5 and 7·5 were investigated using the spectrophotometric guaiacol/peroxidase assay (see Experimental).

Michaelis constant for oxygen

Oxygen concentration was measured by the Clark electrode with a constant initial spermine concentration (10^{-3} M). The O_2 tension was pre-determined before addition of sub-

[11] KUTTAN, R. and RADHAKRISHNAN, A. N. (1972) *Biochem. J.* **127**, 61.

[12] WILLIAMS-ASHMAN, H. G. and SCHENONE, A. (1972) *Biochem. Biophys. Res. Commun.* **46**, 288.

strate by passing O_2 into the reaction solution containing enzyme, buffer and catalase (see Experimental). From the Lineweaver–Burk plot, the K_m for O_2 was found to be $3{\cdot}1 \times 10^{-4}$ M (95% confidence limits $2{\cdot}3 \times 10^{-4}$ M and $4{\cdot}6 \times 10^{-4}$ M), which is close to the O_2 concentration found in air-equilibrated buffer (*ca* $2{\cdot}6 \times 10^{-4}$ M). By saturating the solution with O_2, the rate of spermine oxidation may be doubled, and this was confirmed using the guaiacol/peroxidase system in air-saturated and O_2-saturated incubates. The K_ms for spermine oxidation at two oxygen levels (21 and 80% saturation) were $5{\cdot}5\ (\pm 0{\cdot}4) \times 10^{-5}$ M and $3{\cdot}7\ (\pm 0{\cdot}4) \times 10^{-5}$ M respectively (s.e. in brackets). Since the slope of the lines of the Lineweaver–Burk plot at the two O_2 levels differed significantly ($P < 1\%$) and converged to give similar K_m values, it appears likely that the mechanism for spermine oxidation is "sequential".[14] A ternary complex of O_2, spermine and enzyme may therefore be necessary for spermine oxidation by the polyamine oxidase from barley, unlike the enzyme from pea seedling cotyledons[13] for which the mechanism is "ping pong".[14] In the present experiment the K_ms for spermine at the two oxygen levels were greater than found previously by a factor of about 2. This was probably due to the high level of NaCl (0·1 M) in the incubates. With lower salt levels at 21% O_2 the K_m was $2{\cdot}3 \times 10^{-5}$ M, as determined with the Clark electrode. In earlier work[2] using the guaiacol/peroxidase assay, the K_m for spermine was 3×10^{-5} M at 21% O_2.

TABLE 2. STOICHIOMETRY OF OXYGEN CONSUMPTION AND ALDEHYDE FORMATION FOR PEA AND BARLEY AMINE OXIDASES

Enzyme	Substrate	Aldehyde	Cyclization product	Oxygen consumption in μatom for 1·25 μmol of amine	E_{670} for aldehyde-NBTH-reaction product
Pea seedling	Putrescine	4-Amino-butyraldehyde	1-Pyrroline	1·38	40×10^3
Pea seedling	Spermidine	1-(3-Aminopropyl)-4-aminobutyraldehyde	1-(3-Aminopropyl)-pyrroline	1·29	$12{\cdot}6 \times 10^3$
Barley leaf	Spermine	1-(3-Aminopropyl)-4-aminobutyraldehyde	1-(3-Aminopropyl)-pyrroline	1·34	$12{\cdot}2 \times 10^3$

Oxygen was estimated by the Clark electrode and the aldehydes with the *N*-methyl-2-benzothiazolone hydrazine (NBTH) reagent (see Experimental).

Effect of redox dyes

The similarity of the oxidative mechanism of the barley enzyme to that of the spermidine dehydrogenase of *Serratia*[5] and also the high K_m for oxygen found for the barley enzyme, suggested that the barley enzyme might donate electrons to redox dyes. However, addition of potassium ferricyanide, and of dichlorophenolindophenol with or without phenazine-methyl sulphate (all at 10^{-3} M) to the Clark electrode cuvette had no significant effect on oxygen consumption by the barley leaf enzyme with spermine as substrate. Suzuki and Hirasawa[7] similarly found that the maize leaf polyamine oxidase would not reduce a variety of artificial electron carriers. In the present work, the pea seedling enzyme would not

[13] YAMASAKI, E. F., SWINDELL, R. and REED, D. J. (1970) *Biochemistry* **9**, 1206.
[14] CLELAND, W. W. (1963) *Biochim. Biophys. Acta* **67**, 104.

reduce the redox dyes with putrescine as substrate. This indicates that none of these higher plant enzymes will function as dehydrogenases in the presence of oxygen.

Comparison of the oxygen electrode and the spectrophotometric method for polyamine oxidase assay

Using the oxygen electrode the increment obtained with 50 nmol of putrescine on oxidation by the pea enzyme at maximum sensitivity was equivalent to 500 μV (10 cm on the 1 mV recorder chart). With the same calibration system, the increment (A_{470}) obtained with the spectrophotometric guaiacol/peroxidase system was 0·12 (12 cm on the recorder chart). The sensitivity of the oxygen electrode system was limited by the slope of the 'blank' trace due to oxygen consumption at the electrode surface.[1] In air-saturated buffer total output was 12 mV and this declined at 60 μV/min. Due to the formation of reactive quinones by peroxidative oxidation of the guaiacol, the spectrophotometric method might provide results which are less reliable than those obtained with the oxygen electrode.[2]

Estimation of the aldehyde products

The 3-methyl-2-benzothiazolone hydrazine hydrochloride (MBTH) method of Sawicki *et al.*[15] has been used to estimate the aldehydes produced by plasma amine oxidase,[16] and in the present study the aldehydes 1-(3-aminopropyl)-4-aminobutyraldehyde and 4-aminobutyraldehyde were determined with this reagent. These aldehydes are formed on oxidation of spermine and spermidine respectively by the barley polyamine oxidase and are in equilibrium with respectively 1-(3-aminopropyl)pyrroline and 1-pyrroline. None of the three amines putrescine, spermidine or spermine gave a colour with this reagent at twice the concentration used in the assay. However, the colour obtained with the aldehydes was suppressed by a variety of inorganic and organic anions, including citrate, acetate, phthalate and phosphate. Buffers at pH 4·5–4·8 containing these anions at 0·1 M suppressed the final colour by 80–90%, though no suppression was caused by Tris–HCl buffer (pH 7·5). Neither HCl (0·1 M) nor NaCl (1 M) caused colour suppression. In order to detect the aldehydes in the reaction mixture after amine oxidation, the anions were removed with Dowex 1. A high blank was experienced with the pea extracts, and this was overcome by reducing the amount of enzyme in the incubate.

The close agreement between the extinction coefficients for the aldehydes formed respectively from spermidine by the pea enzyme ($E_{670} = 12{\cdot}6 \times 10^3$) and from spermine by the barley enzyme ($E_{670} = 12{\cdot}2 \times 10^3$) (Table 2) is further evidence for the stoichiometry of these two enzyme systems, shown earlier by GLC estimation of the corresponding cyclic amine 1-(3-aminopropyl)pyrroline.[17] The extinction coefficient for the aldehyde derived from the oxidation of putrescine (4-aminobutyraldehyde) is about 3·5× greater than for 1-(3-aminopropyl)-4-aminobutyraldehyde, and acetaldehyde was found to have an E_{670} of 71×10^3, i.e. about 6× greater. Sawicki *et al.*[15] give the E_{666} for acetaldehyde as 51×10^3. The results for oxygen consumption overall were supra-stoichiometric by about 6%. This could be accounted for by loss of hydrogen peroxide in peroxidative reactions.

1-Pyrroline may be estimated with *o*-aminobenzaldehyde as the yellow quinazolinium complex,[18,19] but pyrroline has not hitherto been separated by GLC. Although 1-(3-

[15] SAWICKI, E., HAUSER, T. R., STANLEY, T. W. and ELBERT, W. (1961) *Anal. Chem.* **33**, 93.
[16] BACHRACH, U. and RECHES, B. (1966) *Anal. Biochem.* **17**, 38.
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[19] MANN, P. J. G. and SMITHIES, W. R. (1955) *Biochem. J.* **61**, 89.

aminopropyl)pyrroline can be separated by GLC, this substituted pyrroline will not react with *o*-aminobenzaldehyde.[17] The NBTH reagent provides a means for estimating both aminoaldehydes at high sensitivity.

Polyamine oxidase activity in various tissues in relation to plant age

After 1–4 and 11 weeks' growth the polyamine oxidase activities in the green laminae were respectively 0·18, 0·33, 0·57, 0·72 and 0·014 units/g fr. wt and 2·1, 3·5, 4·2, 6·9 and 0·07 units/g dry wt. In 14-day-old seedlings, the ratios of activities in the roots, grain (unsown), petiole (colourless stem and lower leaf sheath), lower leaf and upper leaf were respectively 13:16:130:100:61 (activity of lower leaf = 100%). In 11-week-old plants activities in stem (including leaf sheaths) and in immature grain were respectively 0·07 and 0·003 units/g fr. wt.

Subcellular distribution

An earlier experiment[17] indicated that polyamine oxidase activity was associated primarily with a particulate fraction sedimenting at low centrifugal fields (1000*g*), and that activity associated with cell wall material was insignificant. However, the presence of the insoluble cell wall material during the enzyme assay complicated the interpretation of this experiment, since under these circumstances the spermine added as substrate may have been adsorbed to the carboxyl groups in the pectic acid and become unavailable for the enzyme, leading to an apparently low value for the activity of this fraction. In order to overcome this, in the present work activity of sodium chloride eluates of the particulate and cell wall fractions were estimated.

In four determinations the mean ratio of distribution of total activity in supernatant: particles: cell walls was 0·6:2·6:1·0. The ratio for total chlorophyll in these three fractions was 0:4·3:1·0, and extraction as estimated by chloroplast release was therefore about 80% efficient. Activity retained on the muslin used for filtration was less than 5% of the total. Since most cell wall material was retained by the muslin, activity is mainly associated with the particulate fraction. The ratio of activity in particles to cell wall material may range from 12:1 to 3·2:1 depending on whether the NaCl respectively removes or does not remove the enzyme in the unfractured cells found in the cell wall material. In previous work[17] this ratio was 3:1. Even so, in the present study the activity retained by the cell walls is twice as great as would be expected on the basis of incomplete extraction.

EXPERIMENTAL

Materials. Pea cotyledon diamine oxidase and barley leaf polyamine oxidase were prepared as before.[2] The barley leaf enzyme was used without prior dialysis to remove NaCl. The substrates and inhibitors[20] were used as the hydrochlorides. Citrate buffer was used at pH 4·5 and Tris–HCl buffer at pH 7·5 and 7·8.

Enzyme assay. O_2 uptake was measured with a Clark electrode which fed a 1 mV recorder through a potential divider.[21] The electrode cuvette held 2·5 ml. For O_2 saturation, O_2 gas was bubbled for 2 min. Extending this time to 10 min gave no loss of enzyme activity. The spectrophotometric guaiacol–peroxidase method for H_2O_2 production[2] was also used. Protein precipitation on adding the polyamines and on diluting NaCl-containing preparations of the barley leaf enzyme created slight turbidity which increased with time and complicated the spectrophotometric estimation. Corrections were applied for this error. All activities were determined at 25°. The K_i values were determined as before.[2]

Determination of amino aldehydes. Pea extract (0·1 ml) or barley extract (0·2 ml) was placed in the cuvette of the Clark electrode with 0·1 ml of catalase and 2·25 ml of pH 7·5 0·1 M Tris–HCl buffer (pea), or 2·15 ml of pH 4·5 0·1 M citrate buffer (barley). After saturation with O_2, 50 μl of 25 mM substrate was added, and the increment

[20] BROWN, D. and WOODCOCK, D. (1973) *Pest. Sci.* **4**, 485.
[21] RIKMENSPOEL, R. (1969) *Anal. Biochem.* **30**, 292.

of O_2 consumption was determined. The entire sample was then shaken with 0·5 ml (wet vol.) of Dowex 1 × 4 (OH^- form) for 5 min, and 0·5 ml samples of the supernatant were added to 0·5 ml of 24 mM NBTH. After 30 min at 25°, 2·5 ml of 75 mM $FeCl_3$ was added, and 10 min later the vol. was made up to 10 ml with acetone. After centrifuging (2000 *g*, for 5 min), $A_{670\ nm}$ was determined, which was the λ_{max} for the three aldehydes studied. The values were corrected by subtraction of the blanks, in which amine was omitted. Good recovery of known quantities of acetaldehyde added to the blanks indicated that the resin treatment eliminated the suppression of colour formation due to the presence of citrate.

Enzyme activity was estimated in the various tissues of barley plants grown for up to 4 weeks at 22° under fluorescent lamps (11000 lx, 16 hr day) and up to 11 weeks in a greenhouse. Duplicate samples (10 g), which had not been pre-frozen, were macerated in a cooled Virtis homogenizer in 40 ml 0·5 M NaCl in 0·1 M citrate buffer (pH 4·5). After standing for 30 min and centrifuging at 25000 *g* for 5 min at 0°, 0·2 ml of the supernatant was assayed by the guaiacol–peroxidase method. Samples (5 g) of the leaves were taken for dry weight estimations. For the determination of absolute activity, the recorder was calibrated (A_{470}/μmol substrate) by completely oxidizing 30 nmol of putrescine with the pea seedling enzyme (A = ca 0·12). One unit of activity was defined as that amount which would catalyse the transformation of 1 μmol of substrate/min under the conditions of the assay.

Subcellular distribution. Frozen leaf samples (4 samples; 10 g each) of 15-day-old seedlings were macerated with 20 ml H_2O for 3 min in the Virtis homogenizer. On filtration through muslin, the filtrate was frozen for 1 hr and centrifuged (3000 *g* for 15 min) on thawing. The sediment and the cell wall material retained by the muslin were washed in 0·5 M NaCl (pH 4·5) making up the vol. of each to 30 ml. After 16 hr at 2°, extracts were recentrifuged and activity of 0·2 ml aliquots of each sample was determined without removal of the NaCl which is without effect on the assay at this concentration. Chlorophyll estimates (A_{450}) were also made on each fraction in order to determine the degree of breakage of the cells. Since protein precipitation occurred on diluting the supernatant, 0·5 ml of the extract was diluted with 5 ml of buffer and centrifuged after 30 min at 25°. 2·2 ml of this soln was used for assay.

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