THE UPTAKE OF ADENINE BY BREWERS' YEAST AND A RESPIRATORY-DEFICIENT MUTANT

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SUMMARY

On suspending "resting" cells of a strain of brewing yeast and of a derived respiratory-deficient mutant in an adenine-containing medium, there resulted no ultimate concentration of free adenine within the cells although the total concentration of adenine derivatives increased when glucose or certain other carbohydrates were incorporated into the medium. The increase resulted from a stimulation of the conversion of the adenine into nucleotides. After keeping in the presence of adenine and glucose and then transferring to a medium free of glucose, the yeasts released hypoxanthine and not adenine, even though adenine was present within the cells in a form soluble in cold trichloroacetic acid. There was no evidence that the uptake of adenine by these yeasts was dependent upon a specific concentrating mechanism as is the case for certain carbohydrates. It appears rather that the linkage between uptake of adenine and glucose metabolism is due to combination of the purine with a dissimilation product of glucose, possibly 5-phosphoribosyl-1-pyrophosphate, to form nucleotides.

INTRODUCTION

Many yeasts utilize adenine[1,2] but only recently have the metabolic processes involved received detailed study. CHANTRENNE AND DEVREUX[3,4], using isotopic techniques, demonstrated the conversion of adenine into hypoxanthine and other purines by non-proliferating or "resting" cells of certain yeast strains whilst HARRIS AND NEAL[5] have described the products derived from [8-^{14}C]adenine which are present in ethanol and TCA extracts of a brewing yeast at various stages of growth. COWIE AND BOLTON[6] showed that the adenine present under certain conditions in large amounts in the fraction of *Candida utilis* soluble in cold TCA was not freely diffusible. This suggested that a specific concentrating mechanism for adenine might be present in the cell wall of this species and, indeed, later findings[7] provided additional evidence in favour of this concept. That the passage of purines, pyrimidines and nucleotides across some cell membranes may be an energy-linked process has also been suggested as a result of studies on the small intestine of the rat[8,9] and on *Escherichia coli*[10]. Information is lacking, however, as to whether the uptake of adenine by brewers' yeast involves such a system. To investigate this possibility, the uptake, utilization and

Abbreviations: TCA, trichloroacetic acid; PRPP, 5-phosphoribosyl 1-pyrophosphate.

release, over short time periods, of adenine and its derivatives by resting cells of a brewers' yeast and of a derived respiratory deficient mutant strain have been studied.

MATERIALS AND METHODS

Culture of yeasts

Saccharomyces cerevisiae (No. 240, British National Collection of Yeast Cultures) was grown aerobically by shaking at 25° in a medium consisting of yeast extract (Difco, 0.3%), malt extract (0.3%), bacteriological peptone (0.5%) and glucose (1%) dissolved in water. A subculture of the yeast was grown in the same medium (10 ml) for 24 h and used to inoculate 400 ml of the medium on which the yeast was allowed to grow for a further 40 h. The yeast was harvested by centrifugation, washed three times with water and finally suspended in water before use. All the following work was carried out with this yeast unless otherwise stated. A respiratory deficient mutant (240A2) was kindly supplied by Dr. J. W. MILLBANK who obtained it from the first strain using the procedure described by MILLBANK AND HOUGH[11]. The mutant was grown in the same way except that, in view of its relatively slow growth, the first subculture was allowed to develop for 48 h before being used to inoculate the larger batch of medium. After each culture of the mutant the absence of the parent strain was checked by demonstrating that glucose failed to stimulate the uptake of oxygen.

Measurement of ^{14}C accumulation by the yeasts

Samples of yeast (3–3.5 mg dry wt./ml, final concentration) were kept aerobically at 30° in solutions containing phosphate buffer (0.05 *M*) at pH 6.0, adenine ($5 \cdot 10^{-4} M$) and other compounds as appropriate (*cf.*. RESULTS and Tables I–III). Samples (3 or 4 ml) were withdrawn at selected time intervals and the yeast centrifuged off at low temperature. Each sample was washed twice with ice-cold water (10 volumes) and, after the addition of methyl alcohol (0.5 ml), was dried at 50°. The samples were then burnt in a combustion train to carbon dioxide, the radioactivity of which was measured in a gas counter.

Distribution of ^{14}C-labelled materials in yeast extracts after incubating with [8-^{14}C]-adenine

Portions of the above suspensions of yeast were filtered through pads of acid-washed kieselguhr and the collected yeast was washed very rapidly with ice-cold water. The mixtures of kieselguhr and yeast were then transferred individually to either cold 5% TCA, which was immediately frozen, or cold 50% ethanol. The mixture of yeast and TCA was allowed just to thaw, the yeast removed by centrifugation and filtration, and a large proportion of the TCA present in the clear liquid was immediately removed by ether extraction. The remaining TCA was removed by continuous extraction with ether for 40 h. The resulting solution was then concentrated by distillation *in vacuo* at room temperature.

The 50% ethanolic extracts were heated at 50° for a brief period and the residues filtered off. These were extracted twice more with warm 50% ethanol and the combined filtrates in each case concentrated as before. The concentrates were examined by two-dimensional ascending chromatography using the solvent systems (a) *n*-butanol–acetic acid–water (4:1:1, v/v) and (b) *n*-butanol–2.0 *N* ammonia–ethanol[12] (20:5:2,

v/v). Radioautograms were prepared by exposing the sheets to Kodirex X-ray films (Code No. 1) which were developed in the usual way. The ^{14}C-labelled materials were identified by their positions on the chromatograms and by their ultraviolet absorption spectra.

Release of ^{14}C-labelled materials from yeasts

Yeast (3–3.5 mg dry wt./ml, final concentration) was kept in phosphate buffer at pH 6.0 (0.016 *M*), glucose (0.1 %) and [8-^{14}C]adenine ($4 \cdot 10^{-4}$ *M*) for 2.5 h at 30°. The yeast was centrifuged off, washed twice with ice-cold water, suspended in water and finally added to a series of solutions of graded compositions (*cf.* RESULTS and Table IV). The suspensions were then kept aerobically at 30° with shaking. At selected time intervals portions of the mixtures were taken and the yeast removed by centrifugation. A portion of each of the supernatant liquids was then added to powdered cellulose and the mixtures burnt to carbon dioxide for measuring radioactivity as before. Portions of the supernatant solutions were concentrated by distillation *in vacuo* at room temperature and the ^{14}C-labelled compounds present in each concentrate identified by autoradiography.

Chemicals

[8-^{14}C]Adenine was obtained from the Radiochemical Centre, Amersham, Bucks. Other chemicals were of A.R. grade whenever possible.

Analytical methods

Oxygen uptake measurements were made by means of the conventional Warburg apparatus[13]. The utilization of glucose by the yeast was followed by the NELSON[14] modification of the SOMOGYI[15] procedure. Ultraviolet absorption measurements were made on a Uvispek spectrophotometer.

RESULTS

In the initial work in which yeast was shaken in the presence of [8-^{14}C]adenine and phosphate buffer, determination of the distribution of labelled compounds soluble in cold TCA after 2, 5 and 15 min incubation showed that [^{14}C]nucleotides were detected before [^{14}C]adenine. The use of [^{14}C]adenine throughout incubation had previously shown that after 2 h under the same conditions only 20 % of the ^{14}C-labelled material soluble in TCA consisted of free adenine. It was possible therefore that adenine was transformed into nucleotide during entry into the cell. If this were so the free adenine which appeared in the later samples might have arisen either by direct entry or by breakdown of nucleotides. To investigate this last point, yeast (3 mg dry wt./ml, final concentration) was kept for the comparatively long period of 2 h in a solution containing phosphate buffer (0.016 *M*) and adenine ($8 \cdot 10^{-4}$ *M*). A small quantity of [8-^{14}C]adenine was then added to the suspension and the distribution of radioactivity among the compounds soluble in cold TCA examined after selected time intervals. After 2 min the only ^{14}C-labelled material present was adenine whilst after 5 min adenine and hypoxanthine contained considerable labelling and nucleotides were labelled lightly, suggesting that at the later stages of the original incubation at least, adenine is the precursor of the other materials. Assuming that TCA extracts all nucleotides, including those possibly on the cell wall, it is unlikely, therefore, that

transformation to nucleotide is a necessary step in the penetration of adenine into the pool of substances soluble in TCA.

Factors influencing the rate of uptake of adenine by the yeast were investigated. Each of a series of compounds, on which the yeast was able to grow as sole carbon source, was added to the incubating mixture and the amount of radioactivity taken up by the yeast after different time intervals estimated (Table I). It is clear that several compounds are able to stimulate adenine uptake but the greatest effects are shown by sucrose and glucose.

TABLE I

ADENINE UPTAKE IN PRESENCE OF ADDED SUBSTANCES

Uptake in μmoles/g dry wt. of cells. Concentration of added substances, 0.125%, w/v.

Addition	*Time (min)*		
	10	*25*	*60*
None	0.87	1.02	1.26
Maltose	1.35	2.80	—
Galactose	0.61	0.81	—
Sodium acetate	1.50	2.56	—
Sucrose	2.75	5.28	—
Glucose	1.09	4.78	6.50

If the stimulatory effect of the carbohydrates was due to an effect on the rate of penetration of adenine through the yeast cell wall by means for example of a permease, this rate would be independent of the concentration of adenine[16] in the pool of compounds soluble in cold TCA. Accordingly, yeast (final concentration, 3 mg dry wt. of cells/ml) was kept for 2.5 h in: (a) a solution containing adenine ($6.7 \cdot 10^{-4}$ *M*), glucose (0.125%, w/v) and phosphate buffer (0.037 *M*) of pH 6.0 and (b) a solution containing only phosphate buffer (0.037 *M*) of pH 6.0.

The yeasts were removed by centrifugation, washed quickly in cold water and a portion of each taken for a comparison of the contents of free adenine soluble in cold TCA. For this purpose, a small quantity of [8-^{14}C]adenine was added to both suspensions, each suspension was placed immediately in cold 5% TCA and extracts were prepared and developed by chromatography as before. The adenine zones on the resulting chromatograms were detected by autoradiography, and the adenine content of each estimated by elution with water and measurement of the ultraviolet absorption at 260 mμ. The amount of adenine present in the yeast incubated under conditions (a)

TABLE II

EFFECT OF PRIOR TREATMENT WITH ADENINE ON ADENINE UPTAKE

Adenine uptake in μmoles/g dry wt. of cells.

	Cells incubated 2.5 h in presence of adenine		*Cells incubated 2.5 h in absence of adenine*	
Time (min)	*10*	*25*	*10*	*25*
Glucose present	0.81	5.12	0.89	5.18
Glucose absent	0.51	0.63	0.59	0.72

was approx. five times that in yeast incubated under conditions (b). The remaining yeast was incubated aerobically at 30° in (a) [^{14}C]adenine ($5 \cdot 10^{-4}$ *M*), glucose (0.125%, w/v) and phosphate buffer at pH 6.0 (0.037 *M*) and in (b) [^{14}C]adenine ($5 \cdot 10^{-4}$ *M*) and phosphate buffer at pH 6.0 (0.037 *M*). The results (Table II) show that the five-fold greater concentration of adenine in the one case had little effect on the rate of adenine uptake either in the presence or in the absence of glucose.

The relationship of the stimulatory effect on adenine uptake to glucose metabolism was studied by adding DNP at different concentrations to the incubating suspensions containing 0.2% (w/v) glucose. The uptake of adenine was determined as before and at the same time the utilization of glucose by the yeast was measured (Table III). It is evident that there is a broad tendency for increasing DNP concentrations to reduce adenine uptake even though overall glucose utilization is stimulated at certain concentrations.

TABLE III

EFFECT OF DNP ON ADENINE UPTAKE AND GLUCOSE UTILIZATION

DNP concentration (M)	*Adenine uptake after 45 min (μmoles/g dry wt. of cells)*	*% stimulation (+) or inhibition (—) of glucose utilization after 45 min*
Nil	5.15	—
$5 \cdot 10^{-4}$	5.28	+ 23
$1 \cdot 10^{-3}$	3.40	+ 16
$2 \cdot 10^{-3}$	1.59	— 24
$5 \cdot 10^{-3}$	0.97	— 63

Release of ^{14}C-labelled compounds from yeasts after incubation with [8-^{14}C]adenine

Yeast (3 mg dry wt. of cells/ml, final concentration) was incubated for 2.5 h in a solution containing [8-^{14}C]adenine ($4 \cdot 10^{-4}$ *M*), glucose (0.1%, w/v) and phosphate buffer (0.016 *M*) at pH 6.0. The yeast was centrifuged off, washed twice with cold water and equal portions were finally suspended at the same concentration in solutions a–d containing: (a) 0.013 *M* phosphate buffer at pH 6.0, (b) 0.013 *M* phosphate buffer at pH 6.0 and 0.16% glucose, (c) 0.013 *M* phosphate buffer at pH 6.0 and $4 \cdot 10^{-5}$ *M* adenine, and (d) 0.013 *M* phosphate buffer at pH 6.0 and $4 \cdot 10^{-4}$ *M* adenine and 0.16% glucose.

The suspensions were kept at 30° and at suitable time intervals portions were taken, the yeast removed by centrifugation, and the ^{14}C content of the supernatant solutions determined (Fig. 1). It is clear that the release of ^{14}C-containing material is largely prevented by the presence of glucose if the solution is free from adenine but not if adenine is present. Examination of aliquot portions of the supernatant solutions by chromatography and subsequent autoradiography showed that hypoxanthine alone was released into solution containing buffer with or without adenine. If glucose and adenine were both present, however, a more complex chromatographic picture was observed although the materials released were not identified. A similar trial using the respiratory deficient mutant showed that hypoxanthine alone was released when the yeast was resuspended in phosphate buffer solution either in the presence or in the absence of adenine.

Glucose was far less effective in reducing the release of ^{14}C-labelled material when DNP was added to the medium. As the results in Table IV show, the presence of DNP increased the rate of release of ^{14}C-labelled material in the presence of phosphate buffer and glucose even at concentrations at which glucose utilization itself was unimpaired. This result recalls that obtained on the effect of DNP on the stimulating effect of glucose on adenine uptake.

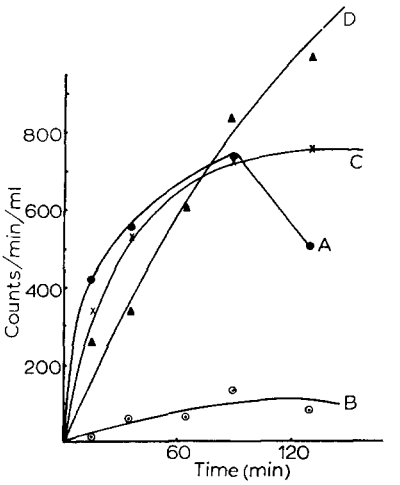

Fig. 1. Release of radioactivity from yeast when suspended in (A) 0.013 *M* phosphate buffer pH 6.0, (B) 0.013 *M* phosphate buffer pH 6.0 and 0.16% (w/v) glucose, (C) 0.013 *M* phosphate buffer pH 6.0 and $4 \cdot 10^{-4}$ *M* adenine, (D) 0.013 *M* phosphate buffer pH 6.0, $4 \cdot 10^{-4}$ *M* adenine and 0.16% (w/v) glucose.

TABLE IV

RELEASE OF RADIOACTIVITY (COUNTS/MIN/ML) FROM THE YEAST WHEN SUSPENDED IN GLUCOSE (0.5%) AND PHOSPHATE BUFFER (0.016 *M*) SOLUTION AND DIFFERING CONCENTRATIONS OF DNP

DNP concentration (M)	*Release of radioactivity counted by end window (counts/min/ml) after 45 min incubation at 30°*
$5 \cdot 10^{-3}$	60
$5 \cdot 10^{-3}$	70
$1 \cdot 10^{-3}$	59
$6 \cdot 10^{-4}$	49
$1 \cdot 10^{-4}$	18
$8 \cdot 10^{-5}$	14
Nil	15

Uptake of adenine by a respiratory deficient mutant (240A2)

The results obtained so far suggested that the uptake of adenine by yeast was related to carbohydrate metabolism. It was felt to be of interest to determine whether a yeast of which the carbohydrate metabolism was very different from that of the brewing strain, for example a derived respiratory deficient mutant, behaved similarly. Investigation of the ^{14}C-labelled materials present in the fraction soluble in cold TCA when the mutant yeast was kept in a solution containing phosphate buffer (0.016 *M*)

and [^{14}C]adenine ($4 \cdot 10^{-4}$ *M*) showed the presence of labelling in both adenine and hypoxanthine but practically none in nucleotides. This provided a means therefore of studying the effect of DNP on the direct uptake of adenine. The effect of glucose on the uptake was also investigated (Table V) and it is clear that glucose markedly stimulates the uptake but that DNP has little effect.

The results demonstrate also that negligible concentration of radioactivity compared with that in the medium takes place when the yeast is suspended in adenine and phosphate buffer alone, the intracellular concentration being calculated on the assumption that the yeast contains 66% of its weight as water[17].

TABLE V

UPTAKE OF ADENINE BY A RESPIRATORY DEFICIENT MUTANT

Adenine uptake in μmoles/g dry wt. of cells.

Addition	*25 min incubation*
None	0.88
Dinitrophenol ($1 \cdot 10^{-2}$ *M*)	0.76
Dinitrophenol ($5 \cdot 10^{-3}$ *M*)	0.80
Dinitrophenol ($2.5 \cdot 10^{-3}$ *M*)	0.84
Glucose (0.125%, w/v)	5.02

Effect of glucose on the incorporation of adenine into nucleotides

After incubation of the yeast for 2 h in the presence of a solution containing phosphate buffer (0.016 *M*), [^{14}C]adenine ($5 \cdot 10^{-4}$ *M*), and glucose (0.125%), examination of the distribution of ^{14}C-labelled material extractable by cold TCA or aqueous ethanol, showed the presence of [^{14}C]nucleotides, adenine, hypoxanthine and a number of other compounds. The effect of subsequent incubation conditions on the proportion of adenine and nucleotide present was also investigated. Yeast was incubated under the above conditions (3.0 mg dry wt. of cells/ml, final concentration), collected

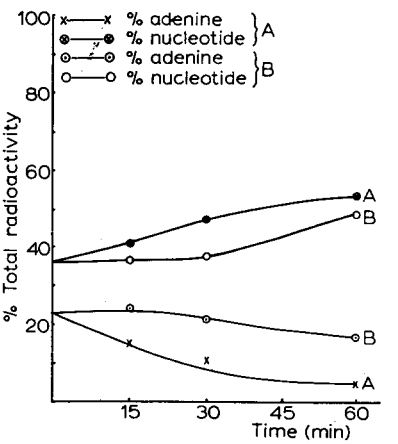

Fig. 2. Distribution of radioactivity between adenine and nucleotide material in (A) glucose (0.1%) and phosphate buffer (0.016 *M*) and (B) phosphate buffer (0.016 *M*).

by centrifugation, washed twice in cold water and resuspended at the same concentration in either: (a) glucose (0.1%) and phosphate buffer (0.016 *M*) solution or (b) phosphate buffer (0.016 *M*) solution alone, and the suspension shaken at 30°. At suitable time intervals portions were removed, filtered through kieselguhr and the yeast extracted as before. The distribution of ^{14}C-labelled materials present in the extracts was examined by chromatography and autoradiography and the radioactivity of the individual zones measured by means of an end-window counter. Typical results are shown in Fig. 2 and similar results have been observed for the ethanolic extracts. Clearly, in the presence of glucose the percentage of the radioactivity corresponding to adenine falls more sharply than in its absence and conversely a more rapid increase of the radioactivity in nucleotides occurs.

DISCUSSION

The results described support the belief that, although the total concentration of soluble adenine derivatives in the cells of brewing yeast may exceed that of adenine in the medium, the uptake of the purine does not involve a specific concentrating mechanism akin to those responsible for the accumulation of di- and trisaccharides[18,19]. The rapidity with which adenine is incorporated into nucleotides and subsequently into nucleic acids[3,5,20] of the normal yeast strains in any event makes difficult the demonstration of the concentration of adenine itself. However, the studies on the respiratory deficient mutant showed that under conditions in which virtually no incorporation into nucleotides or nucleic acid occurred, no concentration of adenine with respect to the external medium took place. Moreover, the dependence of the uptake process upon metabolic energy appears to be excluded by the finding that DNP, at concentrations at which it is an effective uncoupling agent, failed to inhibit the entry of adenine into the mutant. Unless the mutant also loses an energy-requiring concentration mechanism in becoming respiratory deficient it may be concluded that no such mechanism occurs in the parent strain.

Glucose and other sugars nevertheless stimulated the uptake of adenine by both the brewing yeast and the mutant. Presumably, therefore, this effect is due to either a direct chemical linkage between adenine and glucose uptake or an effect by glucose or its derived products on the subsequent metabolism of adenine. The finding that DNP at certain concentrations slightly stimulated the utilization of glucose by the brewing yeast while reducing adenine uptake would appear to rule out the first alternative. Support for the second, however, was the observation that glucose stimulated the conversion of adenine into other substances including nucleotides. Previous investigations have shown the presence in yeasts of enzymes which deaminate adenine to form hypoxanthine[21,22] and of others[23] which catalyze the following reactions:

$$\text{Adenine} + \text{PRPP} \leftrightharpoons \text{AMP} + \text{PP}$$

$$\text{Hypoxanthine} + \text{PRPP} \leftrightharpoons \text{IMP} + \text{PP}$$

Clearly, the rate at which adenine or hypoxanthine is incorporated into nucleotides and later into nucleic acids will depend upon the intracellular concentration of PRPP, and as this is a metabolic product of glucose, the stimulatory effects of glucose become

explicable. The greater percentage increase in the uptake of adenine found after 25 min as compared with 10 min supports the idea that the stimulation by glucose depends upon the formation of greater amounts of a product of glucose metabolism. This concept also explains the inhibitory effect of glucose on the release of ^{14}C-labelled material when the yeast was resuspended in phosphate buffer solution after prior incubation with [8-^{14}C]adenine. An effect of glucose on the proportions of the nucleotides in yeast has already been demonstrated by LAWS AND STICKLAND[24], who observed a rise in the concentrations of ATP and ADP coupled with a reduction in the level of AMP in bakers' yeast during the fermentation of glucose. It appears, therefore, that glucose directly or indirectly increases both the conversion of adenine to AMP and the subsequent phosphorylation of this product. The reversing action of DNP both on the stimulation of adenine uptake by glucose and on the inhibition of the release of ^{14}C-labelled materials when the yeast is suspended in buffer solution also become explicable in terms of the known uncoupling effects of the reagent. Thus, in the presence of DNP at suitable concentrations, glucose utilization can be unimpaired but the resulting net increase in ATP concentration may be lower and in their turn the increase in PRPP concentration and incorporation of adenine into nucleotides.

The finding that only [^{14}C]hypoxanthine was released from the yeast on transfer from a medium containing [8-^{14}C]adenine to phosphate buffer solution with or without adenine is of special interest. This liberation of hypoxanthine finds a parallel in the work of LAHOU[25], who observed that the uptake of guanine by resting cells of a "petite" yeast strain was followed by loss to the medium of the deamination product xanthine. Similarly, the rapid conversion of adenine to hypoxanthine by yeasts has been demonstrated by CHANTRENNE AND DEVREUX[3], who did not, however, separate the yeast from the medium in studying the interconversion. Using bakers' yeast, ROUSH AND SAEED[26] found adenine to be rapidly transformed to hypoxanthine, which was lost from the cells, but adenine itself was not detected intracellularly. The failure of yeast under the conditions used in these investigations to release [^{14}C]nucleotides to the medium, despite their presence within the cells, is in contrast to the results of HIGUCHI AND UEMURA[27] (*cf.* HARRIS AND PARSONS[2]). However, this apparent discrepancy may be due to the differences in the ages of the cultures used, which were 45 h in this work and 80 h in the work of HIGUCHI AND UEMURA. The loss of hypoxanthine by yeast while retaining adenine might be attributable either to the rapid deamination of the latter in transit or to its being not freely diffusible despite the speed with which it is taken up. A finding which mitigates against the first alternative is that under certain of the conditions described under MATERIALS AND METHODS adenine taken up by the yeast appeared only as such in the cell sap. With regard to the second possibility it is relevant to note that in *C. utilis* at least the amount of free adenine in the cell sap can exceed the apparent limits of solubility of adenine[6]. In this instance it seems that adenine must be bound at various sites within the cell and it may be that the phenomenon is of rather frequent occurrence in yeasts.

Relatively large amounts of ^{14}C-labelled material were released from the yeast used in the present investigations when it was suspended in solutions containing phosphate buffer, adenine and glucose but it is not known at present whether [^{14}C]-adenine escaped from the cells under these conditions. This and the reason why the presence of adenine in the external medium reverses the inhibitory effect of glucose on the loss of ^{14}C-labelled materials from the cells requires further investigation.

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