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CONTROL OF RESPIRATION IN DISKS OF CARROT STORAGE TISSUE

T. AP REES and B. J. ROYSTON*

Botany School, University of Cambridge

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Abstract—The aim of this work was to investigate whether the development of induced respiration during the ageing of disks (1 × 10 mm) of storage tissue of carrots (*Daucus carota* L) was accompanied by striking changes in the activity of the tricarboxylic acid cycle The hypothesis that the cycle is inoperative in freshly prepared disks but very active in aged disks was tested The oxygen consumption of fresh and 24-hr aged disks was inhibited by malonate and by arsenite No qualitative difference was found between fresh and 24-hr aged disks in respect of the pattern of $^{14}CO_2$ production from [1-^{14}C]- and [2-^{14}C]-acetate, or in respect of the kinetics of labelling of citric, succinic, and malic acids by [2-^{14}C]-acetate In both fresh and aged disks the labelling patterns were consistent with the operation of the tricarboxylic acid cycle. It is argued that, in carrot storage tissue, the cycle operates in fresh and aged disks and that induced respiration is not caused primarily by a marked increase in the activity of the cycle

INTRODUCTION

CONSIDERABLE changes occur in the metabolism of thin disks of many plant tissues when these disks are incubated under moist conditions at physiological temperatures.[1,2] This phenomenon is called ageing and includes: synthesis of protein,[3] RNA and DNA,[4] an increased ability to metabolize exogenous substrates,[5,6] and a marked increase in the rate of respiration, called induced respiration, that develops gradually over the first 24 hr of ageing.[5] Laties[1,7] has presented considerable evidence that the tricarboxylic acid cycle is virtually inactive in freshly prepared disks of potato tuber but is very active in aged disks. A recent paper[8] from Laties' laboratory contains additional evidence for this view, and the authors have argued that induced respiration in disks of storage organs differs qualitatively from the respiration of freshly prepared disks. Laties[1] has proposed that 'the change in physiological competence manifested by potato slices with time is the consequence of the development of vigorous oxidative phosphorylation, the latter, in turn being dependent upon the upsurge in activity of the tricarboxylic acid cycle'. Both fresh and aged disks of potato yield mitochondria capable of oxidizing intermediates of the cycle[9] and the low activity of the cycle in fresh disks is attributed to a block between the steps involving citric and α-ketoglutaric

* Deceased 25 July 1967.

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acids.[7] The development of induced respiration is regarded as being due to the escape of some volatile inhibitor during ageing.[1]

The tricarboxylic acid cycle is usually held to play a central role in plant respiration Induced respiration is so widespread[2] that this view of the importance of the cycle may require revision if the mechanism proposed by Laties is found to be a general means of controlling plant respiration. This paper reports experiments that were undertaken to see whether the tricarboxylic acid cycle makes a significant contribution to the respiration of freshly prepared disks of carrot storage tissue, and whether any very marked change in such a contribution occurs during the development of induced respiration. The experimental approach was, in general, similar to that of Laties and involved the use of inhibitors of respiration, and the determination of the distribution of label from [^{14}C]-acetate. Investigations of fresh disks were confined to the period immediately following cutting so as to minimize complications caused by the development of the ageing phenomenon.

RESULTS

Effects of Malonate and Arsenite on Rate of Respiration

Malonate consistently inhibited the oxygen consumption of both fresh and aged disks of carrot (Fig. 1). The degree of inhibition produced by a particular concentration of malonate varied with the time that the carrots had been stored For example, with disks from carrots that had been stored for 5 weeks, 5 mM malonate inhibited the oxygen consumption of fresh and aged disks by 32 per cent and 63 per cent of the control rates, respectively. The corresponding figures for carrots that has been stored for 18 weeks were 9 per cent and 22 per cent. At no time was any qualitative difference in sensitivity to malonate found between

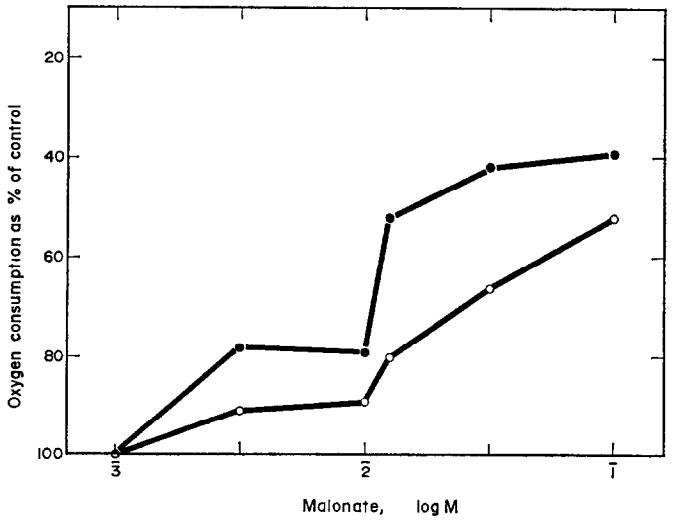

FIG 1 EFFECT OF MALONATE ON OXYGEN CONSUMPTION OF FRESH (○) AND 24-hr AGED (●) DISKS OF CARROT STORAGE TISSUE

Oxygen consumption was measured over a period of 60 min that began 30 min after the addition of the malonate to the disks The malonate was added to fresh disks 45 min after the removal of the first cylinder of tissue. Values are means from a number of experiments in which ageing for 24 hr caused an average increase of 85 per cent in the rate of oxygen consumption

fresh and aged tissue. Any given concentration of malonate inhibited the oxygen consumption of aged disks more than that of corresponding fresh disks. None the less increased inhibition of fresh disks could be obtained by increasing the concentration of malonate (Fig. 1). These observations, and the knowledge that ageing can lead to increased uptake of solutes,[5] suggested that the increased sensitivity of aged disks to malonate might be correlated with increased uptake of malonate. Evidence that this was so is provided by the data in Table 1.

TABLE 1. RELATION BETWEEN UPTAKE OF [2-^{14}C]-MALONATE AND EFFECT OF MALONATE ON OXYGEN CONSUMPTION

Tissue	Treatment	Consumption of oxygen: Rate (μl/hr/20 disks)	Consumption of oxygen: Inhibition (% of control)	Uptake of [2-^{14}C]-malonate (counts/min/20 disks/95 min)
Freshly cut	Control	108		
	5 mM Malonate	88	19	3305
Aged for 24 hr	Control	145		
	5 mM Malonate	91	37	6544

Six replicate samples of fresh disks were cut in 20 min; three of these were incubated for the next 95 min in 2 5 ml 0 02 M KH_2PO_4 (pH 5 2) that contained [2-^{14}C]-malonate (77 530 counts/min) at 5 mM and three in 2 5 ml 0 02 M KH_2PO_4 (pH 5 2) Uptake of [2-^{14}C]-malonate was determined over the whole 95 min; oxygen consumption was determined over the last 75 min of the incubation A similar procedure was used with aged disks All values represent means of data from triplicate samples

Arsenite at 10–20 mM inhibited the oxygen consumption of both fresh and aged disks of carrot by 30–50 per cent of the control rates. No consistent difference in sensitivity to arsenite was found between fresh and aged disks. Table 2 shows that 13·3 mM arsenite not only inhibited oxygen consumption of fresh and aged disks but also simultaneously stimulated CO_2 production.

Metabolism of [^{14}C]-*Acetate*

In short incubations both fresh and aged disks produced $^{14}CO_2$ from [1-^{14}C]- and [2-^{14}C]-acetate (Table 3). The patterns of $^{14}CO_2$ production from fresh and aged disks were similar. There was no instance in which fresh disks failed to release $^{14}CO_2$ from [1-^{14}C]- and [2-^{14}C]-acetate within 20 min of the addition of the [^{14}C]-acetate to the disks. The yield of $^{14}CO_2$ in the first 40 min of the incubation of fresh disks was low. However it is emphasized that triplicate samples were always taken, that agreement between triplicates was good, and that the patterns shown in Table 3 were obtained in eight different experiments carried out with carrots that had been stored for periods ranging from 2–5 months and which gave disks that developed characteristic induced respiration.[5] The $^{14}CO_2$ production by the fresh disks does not appear to be an artifact. Disks that were both prepared and incubated in [1-^{14}C]-acetate under aseptic conditions yielded $^{14}CO_2$ in 40 min in amounts similar to those given in Table 3 Control experiments in the absence of disks showed that the $^{14}CO_2$ production was not a property of the experimental solutions.

Although ageing produced no qualitive changes in $^{14}CO_2$ production, it did lead to a significant increase in the rate at which the $^{14}CO_2$ was released. This increase was also found in disks that had been prepared and aged aseptically. In order to see whether the increased

TABLE 2 EFFECTS OF ARSENITE ON RATES OF RESPIRATION OF FRESH AND 24-hr AGED DISKS OF CARROT STORAGE TISSUE

Tissue	Treatment	Gas exchange (μl/hr/20 disks)	
		Oxygen consumption	CO_2 production
Freshly cut	Control	137	142
	13 3 mM Arsenite	71 (−48%)	173 (+22%)
Aged for 24 hr	Control	202	190
	13 3 mM Arsenite	129 (−36%)	243 (+28%)

Figures in parenthesis represent inhibition or stimulation caused by arsenite as percentage of control values. Rates were measured over a period of 75 min that began 20 min after the addition of the arsenite. The arsenite was added to the fresh disks 40 min after the removal of the first cylinder of tissue.

TABLE 3 PRODUCTION OF $^{14}CO_2$ FROM [1-^{14}C]- AND [2-^{14}C]-ACETATE SUPPLIED TO FRESH AND AGED DISKS OF STORAGE TISSUE OF MATURE CARROTS

Tissue	Position of ^{14}C in [^{14}C]-acetate	Percentage of added ^{14}C recovered as $^{14}CO_2$ in:					
		20	40	60	80	100	120 min*
Freshly cut	1	0 014	0 164	0 594	1 420	2 707	4·130
	2	0 003	0 026	0 086	0·188	0 350	0 611
Aged for 3 hr	1	0 067	0 453	1 458	3 126	5 110	7 43
	2	0 009	0 047	0 154	0·383	0 749	1 19
Aged for 23 hr	1	0 223	1 807	4 330	7 60	10 88	14 63
	2	0 034	0 322	0 953	1 85	3 11	4·74

* Minutes from addition of [^{14}C]-acetate to samples.

All values are means of triplicate samples, each of 30 disks Fresh disks were prepared at 4° in 25 min and were then transferred to reaction vessels at 23° The time between the removal of the first cylinder of tissue and the addition of the [^{14}C]-acetate was 44 min

TABLE 4 PRODUCTION OF $^{14}CO_2$ FROM [1-^{14}C]- AND [2-^{14}C]-ACETATE SUPPLIED TO FRESH AND AGED DISKS OF STORAGE TISSUE OF YOUNG CARROTS

Tissue	Oxygen consumption (μl/hr/25 disks)	Position of ^{14}C in [^{14}C]-acetate	Percentage of added ^{14}C recovered as $^{14}CO_2$ in:					
			20	40	60	80	100	120 min*
Freshly cut	224	1	0 017	0 187	0 676	1 530	2 670	3 880
		2	0 009	0 035	0 107	0 255	0 491	0 952
Aged for 24 hr	234	1	0 196	1 520	4 82	8 76	12 84	16 14
		2	0 032	0 329	1 22	2 86	4 83	6 84

* Minutes from addition of [^{14}C]-acetate to samples

All values are means of triplicate samples $^{14}CO_2$ production was determined with samples of 35 disks Fresh disks were prepared at 4° in 33 min and were then transferred to reaction vessels at 23° The time between the removal of the first cylinder of tissue and the addition of the [^{14}C]-acetate was 47 min

production of $^{14}CO_2$ was associated with induced respiration, [^{14}C]-acetate was supplied to fresh and aged disks of young carrots that do not develop an induced respiration.[4] From Table 4 it can be seen that ageing these disks of young carrots led to increased production of $^{14}CO_2$ despite the fact that no induced respiration developed. Comparison of Tables 3 and 4 shows that the yields of $^{14}CO_2$ from fresh and aged disks of young carrots were, respectively, almost identical to the yields from fresh and aged disks of mature carrots.

TABLE 5. LABELLING OF ACIDS AND CO_2 BY [2-^{14}C]-ACETATE SUPPLIED TO FRESH AND 24-hr AGED DISKS OF CARROT STORAGE TISSUE

		Min from addition of [^{14}C]-acetate	Radioactivity							
			5		10		20		40	
Tissue	Compound		counts/min	counts/min/μmole	counts/min	counts/min/μmole	counts/min	counts/min/μmole	counts/min	counts/min/μmole
Freshly cut	Citrate		897	94	1842	197	4140	441	4914	532
	Succinate		192	73	367	139	596	226	1027	388
	Malate		34	1	133	5	755	25	1295	44
	CO_2		44	—	62	—	119	—	321	—
Aged for 24 hr	Citrate		2255	278	4530	560	7840	968	10,995	1360
	Succinate		226	100	1329	590	1528	678	3960	1765
	Malate		363	14	1422	54	4933	186	10,285	390
	CO_2		48	—	87	—	155	—	538	—

Fresh disks were prepared at 4° in 10 min and were then transferred to reaction vessels at 23°. The time between the removal of the first cylinder of tissue and the addition of [^{14}C]-acetate was 20 min

The kinetics of the labelling of intermediates of the tricarboxylic acid cycle by [2-^{14}C]-acetate are a more stringent test of whether the cycle is operating than are the patterns of $^{14}CO_2$ production. Kinetic studies of this type were undertaken with fresh and 24-hr aged disks (Table 5). No qualitative differences were detected between fresh and aged disks. In both types of disk the rates of labelling and the specific activities of the acids indicate that they were labelled in the order citric, succinic, malic. The results reveal no evidence of a block between citric acid and succinic acid. Finally the data for fresh disks show that label from [2-^{14}C]-acetate not only appeared in CO_2 within 5 min but also labelled three intermediates of the tricarboxylic acid cycle in that time.

DISCUSSION

None of our experiments revealed any qualitative differences in respiratory metabolism between fresh and aged disks. The sensitivity of oxygen consumption to malonate and to arsenite is evidence that the tricarboxylic acid cycle makes substantial contributions to the respiration of both fresh and aged disks. The stimulation of CO_2 production by arsenite would be expected if pyruvate oxidation via the tricarboxylic acid cycle were inhibited.[10] The kinetics of labelling of CO_2, and of citric, succinic and malic acids by [^{14}C]-acetate are consistent with the operation of the cycle in both fresh and aged disks. These labelling patterns

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are closely comparable to those reported for a number of other plant tissues.[11,12] The labelling of citric and malic acids in our experiments is similar to that reported for carrot storage tissue by MacLennan, Beevers and Harley.[12] These authors provided further evidence that this labelling pattern results from the operation of the tricarboxylic acid cycle by determining the distribution of label within both malic and glutamic acids. Taken together the above results constitute strong evidence that the tricarboxylic acid cycle mediates appreciable fractions of the respiration of both fresh and aged disks of carrot storage tissue.

The speed with which we prepared fresh disks, and the appearance of label in intermediates of the cycle within 5 min of adding [^{14}C]-acetate to fresh disks, make it unlikely that qualitative changes in respiration, leading to induced respiration, had occurred before we started our experiments.[1] The results reported in this, and in other papers,[5,13-15] provide no evidence for the occurrence of any qualitative changes in respiration during ageing of carrot storage tissue. On the contrary, the results strongly support the view that glycolysis, the pentose phosphate pathway, and the tricarboxylic acid cycle, all make appreciable contributions to respiration in both fresh and aged disks of carrot.

Our results might be taken to suggest that ageing in carrots is accompanied by a very marked increase in the proportion of respiration that is mediated by the tricarboxylic acid cycle. This question can not be resolved until reliable techniques for assessing the activity of the cycle have been developed. Careful examination of our results shows that they provide little support for the above view. The greater effect of malonate on aged disks could be due to the increased uptake of malonate and to a more severe limitation of respiration by succinic dehydrogenase in the more rapidly respiring aged disks. It is noted that sensitivity to arsenite did not increase appreciably during ageing. There is no doubt that [^{14}C]-acetate labelled CO_2 and the intermediates of the cycle more rapidly in aged than in fresh disks. This does not prove that there was a corresponding increase in the activity of the cycle. The results may merely reflect a more ready access of exogenous acetate to the cycle in aged than in fresh disks. Such access could be altered by changes, in the uptake of acetate from the external solution, in the movement of acetate carbon into the mitochondria, in the activation of acetate, in the compartmentation of acetate within the cell, and in the distribution of acetate between different metabolic pathways. Finally it is pointed out that regardless of the explanation of the increased labelling of the CO_2, our results show that this increase is not specifically associated with the development of induced respiration.

Laties' explanation of induced respiration may not, at present, be applied to carrot storage tissue. The following hypothesis is presented for carrots. Differentiation in plant tissues is probably regulated, at least in part, by gradients of growth substances, such as auxins, cytokinins, gibberellins and ethylene and nutrients, such as sucrose.[16-18] It is proposed that slicing and ageing of mature carrots alter the existing gradients of growth substances and nutrients and that these alterations lead to a stimulation of the non-growing cells to a period of active synthesis resembling growth Net synthesis of protein,[3,19] RNA

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and DNA[4] occurs during ageing of carrot tissue. The development of induced respiration is, to a significant extent, dependent upon these syntheses of RNA and protein.[4] Renewed synthetic activity could lead to the development of induced respiration in two ways. First, increased demands for ATP and reducing power could increase the activity of respiratory enzymes present in fresh disks. The response of fresh and aged disks to 2,4-dinitrophenol,[5] and the recent work of Adams[15,20] and Rowan,[14] strongly suggest that the use of ATP during ageing of carrot tissue leads to increased glycolysis. There is also evidence that the pentose phosphate pathway in fresh disks is limited by the availability of $NADP^+$.[13] The second way in which renewed synthesis could contribute to the development of induced respiration is via a net synthesis of respiratory enzymes. In carrots the activity of one enzyme involved in carbohydrate breakdown, invertase, has already been shown to rise spectacularly during ageing.[21] The essential feature of this argument is that induced respiration is held to be the result of increased synthesis caused by slicing and ageing and not *vice versa* as suggested by Laties.[1]

Despite Laties' work with potato and chicory,[1] the above hypothesis could be generally applicable to the wide range of tissues that show induced respiration. Although the respiration of fresh disks of potato differs markedly from that of aged disks, the behaviour of the fresh disks may be an immediate consequence of cellular disorganization caused by wounding and manipulation during preparation. The work of Jacobson *et al.*[8] strongly suggests that the respiratory substrates in the intact potato are similar to those of aged disks but differ markedly from those of fresh disks. There is good evidence that the tricarboxylic acid cycle makes appreciable contributions to the respiration of both aged disks of potato[7] and intact tubers.[22,23] Thus the low activity of the cycle in fresh disks of potato may represent a temporary and anomalous situation rather than any fundamental mechanism of respiratory control. This view is supported by studies with beetroot that indicate that slicing causes immediate changes in the organization of the endoplasmic reticulum and that these changes are reversed during ageing.[24] Different tissues may vary in the extent to which they are disorganized by cutting but may be similar in that once this disorganization is made good the basic response to slicing is renewed synthesis that leads to the development of induced respiration.

EXPERIMENTAL

Material

Carrots (*Daucus carota* L.) were bought locally and were used at once Young carrots are defined as roots harvested less than 14 weeks from planting in the open and having a maximum diameter of 12 mm. The mature carrots were fully grown and had been stored for periods ranging from 0–5 months. Cylinders of storage tissue were taken vertically and sliced into disks (1 × 10 mm). Disks were cut, rinsed thoroughly in distilled water, and sampled, at 4°. The disks were then transferred to room temperature and put in the experimental vessels. The time between the removal of the first cylinder of tissue and the begining of the experimental treatments of freshly cut disks was never more than 50 min. Disks were aged by gentle circulation in aerated distilled water at 25°. The methods used in the preparation and incubation of disks under aseptic conditions, and in the detection of contaminants, have been described [25] Comparisons between fresh and aged tissue were made only within the same batch of replicate samples.

All [^{14}C]-labelled compounds were obtained from the Radiochemical Centre, Amersham, Bucks.

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Measurement of Gas Exchange

Gas exchange was measured manometrically by the direct method at 25°. Samples of 20 or 25 disks were suspended in 2 5 ml 0 02 M KH_2PO_4 (pH 5 2) or in 2 5 ml of solutions of inhibitors dissolved in 0 02 M KH_2PO_4 (pH 5 2) Only freshly prepared solutions of sodium arsenite were used. All measurements for fresh disks were completed before any induced respiration, detectable manometrically, had developed. For the assessment of the effects of inhibitors the control values are measurements made simultaneously on replicate samples suspended in 0 02 M KH_2PO_4 (pH 5 2) All values represent means of rates of triplicate samples

Uptake of [2-^{14}C]-*Malonate*

Samples were prepared and incubated in 5 mM [2-^{14}C]-malonate as described in the text. Uptake was determined by summing the amount of ^{14}C released as $^{14}CO_2$ and the amount retained in the tissue. At the end of the incubation the medium was removed and the disks were rinsed for 2 min in 2 0 ml 5 mM malonate in 0 02 M KH_2PO_4 (pH 5 2). The rinsing was repeated with another 2 0 ml of malonate and the disks were then killed by addition to a mixture of EtOH–$CHCl_3$–7 N HCO_2H (12 5:3) at −30°. The disks were homogenized in water and the homogenate, together with the mixture used to kill the disks, was reduced to dryness under reduced pressure at 30°. The residue was resuspended in water and samples were converted to $Ba^{14}CO_3$ that was assayed for ^{14}C The small amount (0 8–0 9%) of the absorbed [^{14}C]-malonate that was converted to $^{14}CO_2$ by the disks was collected and assayed as described below A control experiment in which [2-^{14}C]-malonate was subjected to the killing and extraction procedures showed that these procedures did not cause detectable loss of radioactivity.

Metabolism of [^{14}C]-*Acetate*

$^{14}CO_2$ production was determined as described previously[26] with samples of 30 or 35 disks suspended in 5 0 ml 0 02 M KH_2PO_4 (pH 5 2) that contained 2 8 μc [1-^{14}C]-acetate or 6 0 μc [2-^{14}C]-acetate at 0 25 mM All data are means of values from triplicate samples.

The labelling of intermediates of the tricarboxylic acid cycle was determined by incubating samples of 30 disks in 5 0 ml 0 033 M KH_2PO_4 (pH 5 2) that contained 6 0 μc [2-^{14}C]-acetate at 0 25 mM. At the end of the incubation the medium was rapidly removed and the disks were killed and extracted in boiling 80% (v/v) aq. ethanol. The disks were then homogenized and the homogenate was extracted successively with 25 ml portions of 20% (v/v) aq ethanol (twice) and boiling water (twice). All the extracts were combined and evaporated under reduced pressure at 30° The residue was extracted with water and known amounts of citric, succinic, and malic acids were added to the solution in order to make it easier to isolate individual radioactive acids. The resulting solution was divided into basic, acidic, and neutral fractions.[13] The acidic components were then fractionated further by gradient elution from 1 × 10 cm columns of Dowex 1 resin (formate) using 8 N HCO_2H in the acid reservoir.[27] Sequentially eluted samples of 2 0 ml were collected, dried in an air stream at 30°, and made up to 2 0 ml with CO_2-free water before titration. This procedure isolated citric acid but did not clearly separate succinic acid from malic acid. Thus the fractions that contained succinic and malic acids were combined, reduced in volume, and run on to a 1 × 10 cm column of Dowex 1 (acetate) Gradient elution was carried out with 8 N HOAc in the acid reservoir. This procedure isolated succinic and malic acids. When mixtures of known amounts of acids were taken through the complete fractionation procedure, recoveries were: citric acid, 94%, succinic acid, 93%; malic acid, 85%. These acids were identified by co-chromatography with known acids on the columns and on paper For determinations of total radioactivity of each acid, all the samples comprising a particular peak were pooled.

Assay of ^{14}C

All measurements of ^{14}C were made after it had been converted to $Ba^{14}CO_3$. The techniques used in this conversion and in the measurement of the radioactivity of the $Ba^{14}CO_3$ have been described.[13] All samples were counted for 4000 sec or until 10,000 counts had been recorded

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