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UTILIZATION OF UREA BY *CANDIDA FLARERI*

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Abstract—The pathway of urea utilization in *Candida flareri* has been investigated using short-term [^{14}C]-urea feeding experiments. It is thought that urea plus a "C_2" fragment gives rise to hydantoic acid which is cleaved to carbamyl phosphate and glycine. The carbamyl phosphate can give rise to carbamyl aspartate, citrulline or carbon dioxide and ammonia. Citrulline may be then metabolized to arginine; the carbon dioxide can be incorporated into oxaloacetic acid which gives rise to aspartic acid and, by the tricarboxylic acid cycle, to glutamic acid. The radioactivity in asparagine, threonine and isoleucine is derived from aspartic acid, and that in glutamine and proline from glutamic acid.

INTRODUCTION

SEVERAL ways have been suggested by which urea may be utilized by organisms which do not contain urease. Walker[1] and Thomas and Krauss[2] have suggested utilization by a reversal of the ornithine cycle. Hattori[3] has postulated an acceptor, A, in *Chlorella* which reacts with either free NH_3 or urea-NH_2 to form A $(NH_2)_2$. Kating,[4] using yeasts, has evidence for a cleavage of urea to a carbamyl residue and ammonia, and Valentine and Wolfe[5] have shown that an extract of *Streptococcus allantoicus* produced glyoxyl-urea when incubated with urea. Reversal of the pathway of purine degradation is another possibility.[6]

The present communication describes the results of short term ^{14}C-urea feeding experiments with *Candida flareri* which has been shown by McEvoy[7] not to contain urease.

RESULTS

Urease activity was not detected in intact cells of *Candida flareri*, in extracts made by disrupting these cells in a Hughes press block, or in the supernatant fluid and residue obtained by centrifugation of these extracts at 20,000 *g* for 20 min.

The extracts did not contain an inhibitor of urease activity since the activities of preparations made by dissolving B.D.H. urease tablets in buffer were unaffected by the addition to them of aliquots of *Candida flareri* extracts. It was shown also that the inactivity of *C. flareri* extracts was not due to the extraction procedure since a preparation of urease (B.D.H.) subjected to the same treatment retained its activity.

The results in Table 1 show that after feeding [^{14}C]-urea to *Candida flareri* for various periods of time, the amount of radioactivity in the anionic fraction of compounds extracted

[1] J. B. WALKER, *Proc. Nat. Acad. Sci. U.S.* **38**, 561 (1952).
[2] W. H. THOMAS and R. W. KRAUSS, *Plant Physiol.* **30**, 113 (1955).
[3] A. HATTORI, *Plant and Cell Physiol.* **1**, 107 (1960).
[4] H. KATING, *Biochem. Z.* **335**, 366 (1962).
[5] R. C. VALENTINE and R. S. WOLFE, *Nature, Lond.* **191**, 925 (1961).
[6] S. R. FREIBERG, E. G. BOLLARD and M. P. HEGARTY, *Plant Physiol.* **32**, lii (1957).
[7] D. MCEVOY, *Studies on the Biosynthesis of Riboflavin in Candida flareri*. University of Liverpool, Ph.D. Thesis (1959).

from the yeast was initially greater than that in the cationic fraction, and also that the total activity of both fractions reached a maximum after 6 min. The non-ionic fraction was probably mainly [^{14}C]-urea, since this compound is not retained by ion-exchange resins under the conditions used. After 0·5 min the greatest amount of radioactivity was in hydantoic acid and

Table 1. Changes with time in the distribution of radioactivity (c.p.m.) in fractions isolated from extracts of *Candida flareri* after feeding [^{14}C] urea

	Time (min)						
Fraction*	0	0·5	1	2	6	10	20
Cationic	0	22	306	764	5119	4430	1894
Anionic	0	494	596	683	1723	1064	625
Non-ionic	9500	8340	7230	5820	2020	550	538

* See Experimental.

its cyclic anhydride hydantoin (Table 2). The radioactivity in different compounds reached a maximum after various feeding times (see Table 2), viz. hydantoic acid, hydantoin and γ-aminobutyric acid after 1 min; ornithine and unknown *1* after 2 min; aspartic acid, asparagine, citrulline, carbamyl aspartic acid, hydantoin-5-acetic acid and unknowns, *2, 4, 5, 6, 8, 9, 10, 11* after 6 min; glutamic acid, argininosuccinic acid, threonine, glutamine, arginine, proline, isoleucine, and unknowns *3* and *7* after 10 min.

DISCUSSION

McEvoy[7] has shown that *Candida flareri* does not contain urease, but nevertheless is able to utilize urea as a sole nitrogen source; these findings have been confirmed.

Initial Entry of [^{14}C]-*Urea*

As a result of short-term feeding experiments with [^{14}C]-urea (see Tables 1 and 2) the pathway of metabolism of urea by *Candida flareri* shown in Fig. 1 is proposed. When [^{14}C]-urea was fed to *C. flareri* for up to 1 min the anionic fraction contained more radioactivity than the cationic fraction (Table 1). Isolation, separation and measurement of the radioactivities of the compounds present in these fractions showed that the two chemically related ureides, hydantoic acid and hydantoin, were more radioactive than any other compounds (Table 2). The results in Table 2 also show that only in these two ureides did the amount of radioactivity decrease after 1 min; the similarity of the amount of radioactivity in these two compounds was probably due to the fact that they were in equilibrium under the conditions used to extract and separate anionic compounds.

Production and Fate of Carbamyl Phosphate

The next step in the proposed pathway (Fig. 1) is the production of carbamyl phosphate and glycine from hydantoic acid, similar to the phosphorolytic cleavage of carbamyloxamic

acid to carbamyl phosphate and oxamic acid which occurs in *Streptococcus allantoicus*.[5] Carbamyl phosphate was not detected, presumably since it is extremely labile,[8] but glycine, although present, was not radioactive as predicted by the pathway. The production of

TABLE 2. CHANGES WITH TIME IN THE RADIOACTIVITY (c.p.m.) OF AMINO ACIDS AND ANIONIC COMPOUNDS OF *Candida flareri*

Compound*	Time of feeding (min)					
	0·5	1	2	6	10	20
Aspartic acid	Tr†	Tr	290	326	508	76
Glutamic acid	Tr	Tr	54	906	924	622
Argininosuccinic acid	Tr	28	92	966	1172	500
Unknown 1 (0·04/0·42)	0	36	92	Tr	Tr	Tr
Asparagine	Tr	Tr	96	101	54	44
Threonine	0	0	18	226	260	166
Glutamine	0	0	0	112	130	Tr
Citrulline	12	48	91	1936	1176	328
γ-Aminobutyric acid	Tr	62	Tr	Tr	Tr	Tr
Arginine	0	18	20	46	134	112
Proline	0	0	0	Tr	40	34
Isoleucine	0	0	0	Tr	22	12
Ornithine	Tr	14	22	0	0	0
Glycine	0	0	0	0	0	0
Serine	0	0	0	0	0	0
Alanine	0	0	0	0	0	0
Valine	0	0	0	0	0	0
Tyrosine	0	0	0	0	0	0
Phenylalanine	0	0	0	0	0	0
Histidine	0	0	0	0	0	0
Lysine	0	0	0	0	0	0
Unknown 2 (0·0/0·07)	39	42	51	85	72	63
„ 3 (0·0/0·14)	46	64	73	74	136	78
„ 4 (0·18/0·15)	0	Tr	35	224	154	92
Carbamyl aspartic acid	15	61	181	756	412	215
Unknown 5 (0·0/0·24)	14	17	23	65	16	12
Hydantoin-5-acetic acid	Tr	Tr	20	137	28	15
Hydantoic acid	150	164	76	11	Tr	0
Unknown 6 (0·4/0·44)	Tr	Tr	Tr	19	15	Tr
„ 7 (0·38/0·63)	Tr	Tr	Tr	35	49	Tr
„ 8 (0·55/0·68)	Tr	Tr	Tr	15	11	Tr
„ 9 (0·20/0·76)	Tr	Tr	Tr	25	22	13
„ 10 (0·29/0·88)	0	0	0	30	0	0
Hydantoin	125	131	72	0	0	0
Unknown 11 (0·44/0·98)	28	31	46	107	63	56

* Figures in brackets refer to R_f values of compounds in EBWA and PW respectively.
† Tr = less than 10 c.p.m.

glycine in this way could explain why Audley, Goodwin and McEvoy[9] found that the carbon of [^{14}C]-urea was not incorporated into riboflavin, even though riboflavin biosynthesis was increased in the presence of urea. Indirect evidence of carbamyl phosphate being involved in

[8] L. SPECTOR, M. E. JONES and F. LIPMANN, in *Methods in Enzymology*, Eds. S. P. COLOWICH and N. O. KAPLAN, 3, 653, Academic Press, New York, (1957).
[9] B. G. AUDLEY, T. W. GOODWIN and D. MCEVOY, D., *Biochem. J.* 72, 8P (1959).

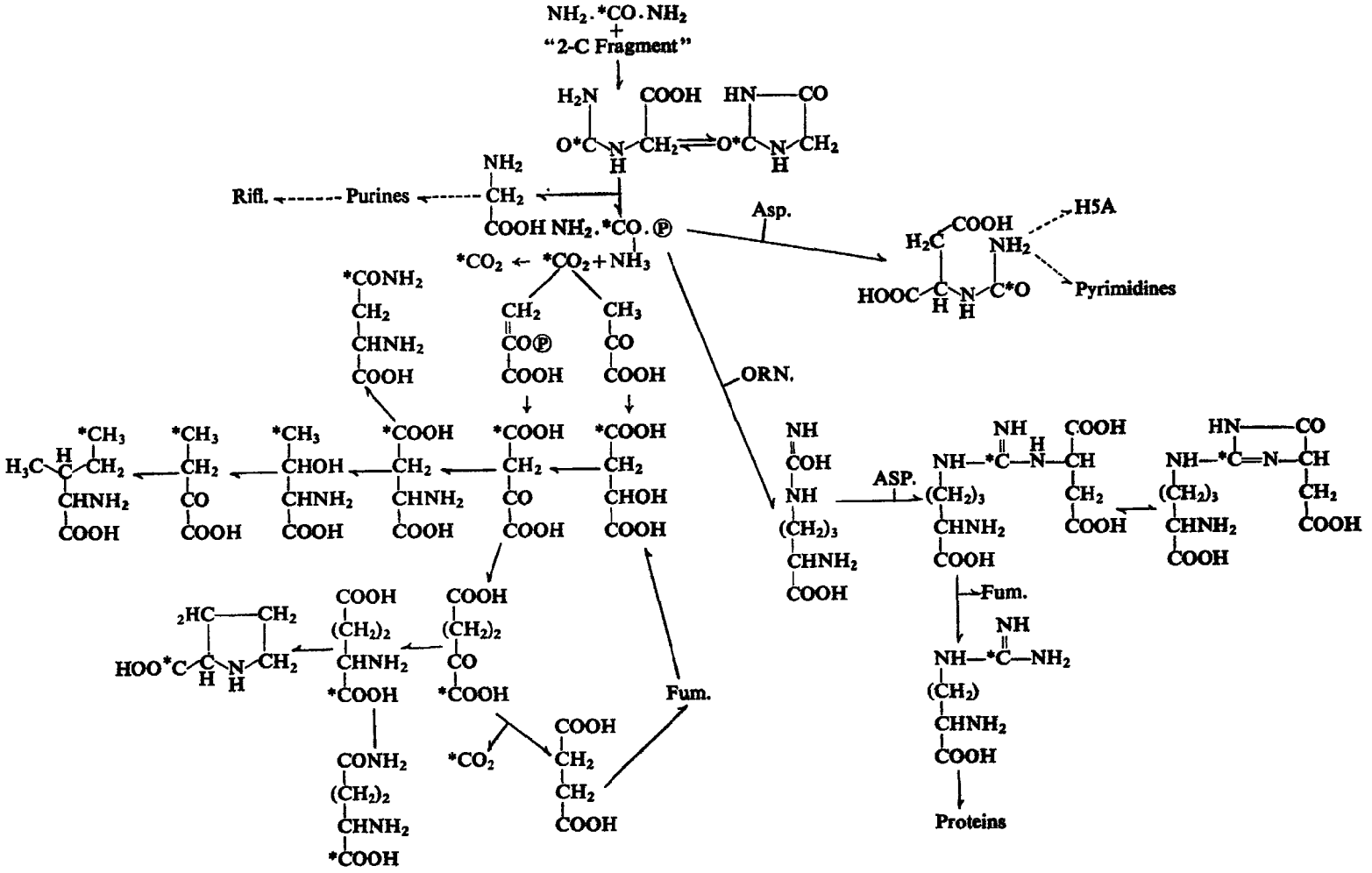

FIG. 1. PROPOSED PATHWAY OF UREA METABOLISM IN *Candida flareri*.

Abbreviations:
ASP,* C, FUM, H5A, ORN, P, RIFL = aspartic acid, radioactive carbon, fumaric acid, hydantoin-5-acetic acid, ornithine, $-OPO_3H_2$, riboflavin respectively.
– – – – – → = pathway postulated from indirect evidence.

the utilization of [^{14}C]-urea comes from a comparison of the amount of radioactivity in citrulline and carbamyl aspartate which indicates that these two compounds had a common precursor. Thus radioactivity was measurable in these compounds after 0·5 min, was at a maximum after 6 min and thereafter decreased. Carbamyl aspartate is known to be synthesized from carbamyl phosphate and aspartic acid[10] and citrulline from carbamyl phosphate and ornithine.[11] A comparison of the radioactivity in aspartic acid and carbamyl aspartate at 0·5 min and 1 min indicates that the radioactivity was mainly in the carbamyl moiety of the carbamyl aspartate.

Metabolism of Citrulline

Radioactivity appeared first in citrulline then in argininosuccinic acid and arginine; it reached a maximum in citrulline at 6 min and in argininosuccinic acid and arginine at 10 min, indicating that these amino acids were in the reaction sequence–citrulline—argininosuccinic acid—arginine. These results may be compared with those of Walker[1] who suggested that the ornithine cycle was reversed in *Chlorella* fed on urea.

Production of Aspartic and Glutamic Acids

After 2 min the radioactivity in aspartic acid was greater than that in glutamic acid; the maximum radioactivity was measured in aspartic acid after 6 min and glutamic acid after 10 min. These acids were most probably formed from their oxo-analogues,[12] and to explain the sequence of labelling in the amino acids it is suggested that radioactive carbon dioxide, formed from the action of carbamate kinase on carbamyl phosphate, reacted with either phosphoenolpyruvic acid to form oxaloacetic acid directly or with pyruvic acid to form malic acid which was subsequently dehydrogenated to oxaloacetic acid. This latter compound either formed aspartic acid by transamination or entered the tri-carboxylic acid cycle to give α-oxoglutaric acid from which glutamic acid was formed by either transamination or direct amination.

Production of Asparagine, Threonine, Isoleucine

The similarity in the "flow" of radioactivity in aspartic acid and asparagine suggests that there was a close biochemical relationship between these compounds; this is in agreement with the results of Webster and Varner.[13] The appearance of radioactivity in threonine later than in aspartic acid suggests that threonine was formed from the latter.[14,15] Radioactivity did not appear in isoleucine until a significant amount of radioactivity had accumulated in threonine and this is in accord with present ideas on the biosynthesis of isoleucine.[16]

The Production of Glutamine and Proline from Glutamic Acid

The similarity in the "flow" of radioactivity in glutamic acid and glutamine suggests that glutamine was formed from glutamic acid.[17] Proline may be formed from either glutamic acid

[10] M. E. JONES, in *Methods in Enzymology*, Eds. S. P. COLOWICK and N. O. KAPLAN, 5, 903, Academic Press, New York (1962).
[11] S. NEWBOLD and P. P. COHEN, unpublished, cited from *Metabolic Pathways*. Ed. D. N. GREENBERG, 2, 56. Academic Press, New York (1961).
[12] G. C. WEBSTER, *Nitrogen Metabolism in Plants*, Row, Peterson and Co., Illinois (1958).
[13] G. C. WEBSTER and J. E. VARNER, *J. biol. Chem.* 215, 91 (1955).
[14] S. BLACK and N. G. WRIGHT, *A Symposium on Amino Acid Metabolism*, Ed. W. D. MCELROY and H. B. GLASS, p. 591, The John Hopkins Press, Baltimore (1959).
[15] Y. WATANABE and K. SHIMURA, *J. Biochem. Tokyo* 43, 283 (1956).
[16] D. M. GREENBERG, in *Metabolic Pathways*, Ed. D. M. GREENBERG, 2, 173, Academic Press, New York (1961).
[17] G. C. WEBSTER, *Plant Physiol.* 29, 382 (1954).

or arginine[15]; the radioactivity data of Table 2 supports the postulated pathway (Fig. 1) for the origin of proline from glutamic acid since radioactivity was detected in glutamic acid after 0·5 min and in proline after 6 min. If arginine had been its precursor, proline would have been unlabelled.

The results in Table 2 show that there were also a number of radioactive anionic compounds whose identity has not been established, and the relationship of these compounds to the pathway of [^{14}C]-utilisation is therefore unknown.

Whilst the existing radioactive data agrees with the proposed scheme (Fig. 1), confirmation must wait further radioactive experiments in which specific activity measurements are made; this will involve the use of larger amounts of material. It will also be necessary to demonstrate the postulated enzymes and show, where possible, their activities *in vivo* by the use of selective inhibitors.

EXPERIMENTAL

Substrates and Reference Compounds

Unless stated otherwise, chemicals were obtained from the British Drug Houses Ltd.; hydantoic acid and hydantoin from The Nutritional Biochemicals Corp., Cleveland, Ohio; hydantoin-5-acetic acid, the K. and K. Labs., Inc., New York; and [^{14}C]-urea from The Radiochemical Centre, Amersham, England.

DL-Carbamyl aspartate (m.p. 175°) was prepared according to Nyc and Mitchell;[18] L-carbamyl glutamate was prepared according to Lowenstein and Cohen[19] and purified using De-Acidite FF resin (formate form), and glyoxyl urea was prepared according to Valentine and Wolfe.[20] The identity of these compounds was established by their R_f values in various solvents. Argininosuccinic acid was separated from an extract of *Candida flareri*. It was eluted from cation exchange resins just before tyrosine and its identity was confirmed by alkaline hydrolysis.[21]

Culture Methods

Candida flareri NRRL 245 (North Regional Research Laboratories, Peoria, Illinois, U.S.A.) was grown in aerated liquid medium of the following composition—20 g glucose; 0·43 g urea (Seitz filtered); 0·5 g KH_2PO_4; 0·5 g $MgSO_4.7H_2O$; 0·3 g $CaCl_2.2H_2O$; 0·1 μg biotin; 70 μg Zn; 10 μg B, Mn, Cu, Mo and Fe; pH of medium adjusted to 5·3.

Feeding Conditions and Extraction and Separation of Radioactive Compounds

The cells were grown in a separating funnel in 150 ml aerated culture medium. 75 μc [^{14}C]-urea were added and 15 ml aliquots withdrawn at 0, 0·5, 1, 2, 6, 10 and 20 min into 15 ml boiling 0·02 M sodium phosphate buffer, pH 7·0. The cells were re-extracted with two changes of boiling buffer and one of boiling 80% (v/v) ethanol and the extracts concentrated *in vacuo* at 35°. Proteins were removed by precipitation with acetone and the extracts de-salted by the method of Harris, Tigane and Hanes.[22] The extract was put on a cation exchange resin (Zeokarb 225) and the anionic and non-ionic compounds washed through with 20 ml H_2O, concentrated to dryness, dissolved in a minimum of water and after adjusting

[18] J. F. NYC and H. K. MITCHELL, *J. Amer. Chem. Soc.* **69**, 1382 (1947).
[19] J. M. LOWENSTEIN and P. P. COHEN, in *Manometric Techniques*, 3rd Ed., Eds. W. W. UMBREIT, R. H. BURRIS and J. F. STAUFFER, p. 305, Burgess Publishing Co., Minneapolis (1957).
[20] R. C. VALENTINE and R. S. WOLFE, *Biochem. biophys. Res. Comm.* **5**, 305 (1961).
[21] S. RATNER, B. PATRACK and O. ROCHOVANSKY, *J. biol. Chem.* **204**, 94 (1953).
[22] C. K. HARRIS, E. TIGANE and C. S. HANES, *Canad. J. Biochem.* **39**, 439 (1961).

the pH to pH 8 put on a Dowex-1 (formate form) column. The non-ionic fraction was washed through with 20 ml H_2O, concentrated to dryness and taken up in a known volume of water. The anionic fraction which remained on the column was then eluted with 12 N formic acid and the eluate made to known volume. The cation fraction which had remained on the Zeokarb 225 column was eluted with 2 N triethylamine in 20% (v/v) acetone and the eluate made to known volume.

The radioactivity of each of these three fractions was determined by measuring the radioactivity of 0·5 ml aliquots placed on tared planchets, containing a disc of lens tissue.

Aliquots were taken for two-dimensional chromatography on Whatman No. 3 MM paper using: (*a*) (EBWA) ethanol, 2-methyl-propan-2-ol, water, ammonia (12:4:3:1, v/v), (*b*) (PW) 80% (w/v) phenol with cyanide and ammonia in the atmosphere. Autoradiograms were prepared using Kodak X-ray film and radioactivities were measured on the paper with a thin end-window G.M. tube (1 in. dia.).[23] Radioactive compounds were identified by eluting them from the paper with either 10% (v/v) isopropyl alcohol (cationic compounds) or 0·02 M sodium phosphate buffer, pH 7·0 (anionic compounds), concentrating, de-salting (in case of anionic compounds) and co-chromatographing them with a suspected known compound.

Urease Activity Measurements

One millilitre aliquots of either a solution of B.D.H. urease tablets or extracts of *Candida flareri* disrupted in Tris/EDTA buffer in a Hughes press block were added to 0·025 M Tris/0·01 M EDTA buffer, pH 8, containing urea and incubated for 5 min at 30°. Any ammonia produced by urease activity was steam-distilled into 5 ml 2% (w/v) boric acid plus four drops of mixed indicator[24] and this solution titrated against 0·1 N HCl (enzyme activities were expressed as millilitres 0·1 N HCl required to titrate formed ammonia).

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[23] J. Glover, in *Modern Methods of Plant Analysis*, Eds. K. Peach and N. V. Tracey, **1**, 325, Springer-Verlag, Berlin (1956).
[24] T. S. Ma and G. Zuazaga, *Industr. Engng. Chem. (Anal).* **14**, 280 (1942).