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THE NATURE OF THE METABOLITES OF FLUOROACETIC ACID IN BAKERS' YEAST

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Abstract—The exposure of cells of bakers' yeast to fluoroacetic acid results in the synthesis of at least two toxic fluoro-fatty acids. Chromatographic characteristics would permit their tentative identification as the fluorinated moieties of butyric and hexanoic acids.

The cells also contain small amounts of unmetabolized fluoroacetate, but repeated analyses failed to detect the presence of fluorocitrate.

DURING an investigation into the nature of the toxic action of fluoroacetic acid (HFA) in cells of bakers' yeast[1, 2] the question arose as to whether or not fluorocitric acid was synthesized from HFA according to the "lethal synthesis" concept of Peters and co-workers.[3]

The fact that acid aqueous extracts of HFA-treated yeast cells do in fact contain a toxic principle was established by Peters, who kindly carried out the assays in his laboratory at Babraham, Cambridge. When injected into rats the extract produced convulsions and alterations in cardiac rate and rhythm. Citrate analyses of the heart, liver and kidney tissues of the poisoned animals, however, showed relative concentrations quite different from those induced by authentic fluorocitric acid.

The purpose of this communication is to report on further work designed to identify the toxic agent elaborated from HFA by viable, but non-dividing yeast cells.

MATERIALS AND METHODS

Preparation of yeast cells

Cakes of commercial (DCL) yeast were obtained from the local manufacturer in Cambridge. Fifty grammes of cells were thoroughly washed with distilled water or M/20 KH_2PO_4 and suspended in 500 ml McIlvaine's citric acid–phosphate buffer, pH 2·1 containing 25 g glucose. After several min stirring, 10 ml HFA solution pH 2·1 was added (final concentration 5×10^{-3} M HFA) and the suspension gently aerated for 30 min. The cells were then centrifuged, resuspended in 500 ml distilled water and re-centrifuged. This washing procedure was repeated and on the third washing the sample was divided into four aliquots containing approximately 12 g cells each. These samples of cell paste were frozen solid and stored in this condition.

Extraction Procedure

A sample of frozen cells representing about 12 g (wet weight) was thawed and alternately film-frozen and thawed twice to render the cells permeable to citrate. Twenty millilitres 8% trichloroacetic acid was added and the cells left to extract

overnight at 5°C. The extract was freed from precipitated protein by centrifugation and freed from trichloroacetic acid by passing steam through the extract until the distillate had become neutral in reaction. Under partial vacuum the volume of the extract was reduced to 5 ml. Yeast "gums" containing a high proportion of phosphate were removed by acidifying the extract to about pH 4·0 with the small volume of HCl and adding acetone to make the final mixture 90 per cent in acetone. After standing at room temperature for 2 hr and shaking occasionally, a flocculent precipitate formed, which was centrifuged down. The supernatant fluid was removed and the acetone blown off under a stream of air. The final volume of the extract was 5 ml.

Column Chromatography

Three millilitres of the extract was evaporated to dryness and the residue (83·9 mg) taken up in 0·5 ml N H_2SO_4. The solution was then applied to a silicic acid column prepared according to Bulen *et al.*[4] by mixing the extract with 1 g silicic acid. Eluting solvents (n-butanol in washed chloroform) were prepared according to Bulen[4] and were run through the column at about 1 ml/min. Three millilitre fractions were collected to which 1 ml distilled water containing phenol red had been added. Titration with 0·01N NaOH was carried out using a Conway micro-burette of 250 μl capacity.

Paper Chromatography

After evaporating off the acetone (see extraction procedure) cations were removed from the extract by mixing 2 ml of it with 2 ml freshly generated Amberlite 120 (H) resin. The strongly acid solution was now separated from the resin and shaken vigorously with an equal volume of 50% n-butanol in chloroform, which had been previously equilibrated against 0·5 N H_2SO_4.

After centrifuging to separate the two phases, 20 μl. from each was spotted on strips of No. 1 Whatman filter paper and chromatographed in n-butanol: 1·5 N NH_4OH. The papers were dried at room temperature, sprayed lightly with brom-cresol purple–formaldehyde–ethanol reagent[5] and, after drying, sprayed with M/200 aqueous borax to visualize the spots.

Aqueous extracts of the butanol–chloroform phase were also prepared by adding an equal volume of water containing phenol red. A solution of NaOH (0·1 N) was added dropwise to the mixture, shaking vigorously between each addition of NaOH until the aqueous phase remained a faint pink. Samples of the aqueous phase were then chromatographed as above. The residual organic phase, when chromatographed, gave rise to no spots, indicating that the acidic material had been completely extracted into the alkaline aqueous phase. This aqueous extract was also used in the toxicity tests.

Fluoride Determinations

Fractions constituting the four major peaks obtained from the silicic acid column analysis were pooled and each evaporated to dryness. To the residues, 0·5 ml 63% aqueous KOH was added and the tubes heated in a boiling water bath for 60 min. Neutralization was carried out by the careful addition, one drop at a time, of concentrated (72 per cent) perchloric acid. The samples were cleared of precipitate by centrifugation and 10μl. solution analyzed for fluoride using a modification of the method of Hall.[6] The colour reactions obtained were compared with that resulting from the analysis of 5μg KF, thereby providing a rough quantitative estimation of the fluoride content of the hydrolyzed extract.

Toxicity Tests

Samples of the aqueous phase and the alkaline water-extracted organic phase obtained as described under "paper chromatography" were carefully neutralized, and 1 ml of each was injected i.p. into female rats weighing about 250 g. Cardiac function was checked at frequent intervals by recording rate and the occurrence of arrhythmias. The general appearance and the behaviour of the animals was also noted. Five hours after injection the animals were killed and the hearts, lungs and kidneys removed for citrate analysis, which were carried out according to the method of Taylor.[7]

RESULTS

Figure 1 depicts the chromatogram obtained from titration of the silicic acid column fractions. According to Bulen *et al.*[4] the second peak from the left should contain acetic acid and therefore it is very likely that the first high peak should contain substances which were not retained by the column, i.e. polar substances highly soluble in chloroform. Such substances might well be aliphatic fatty acids.

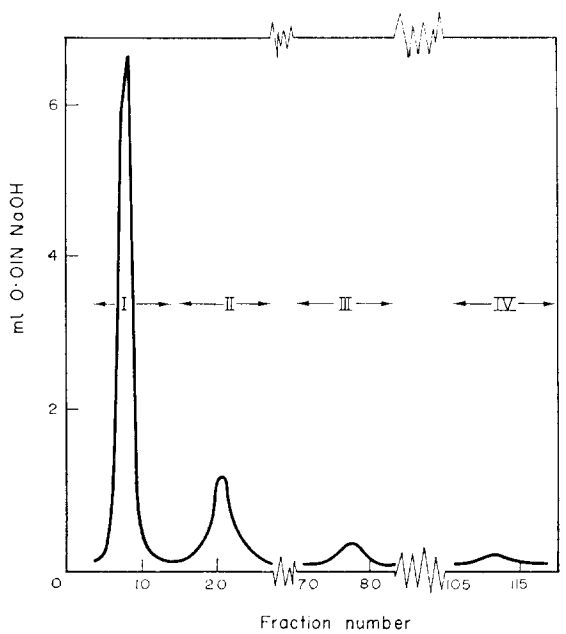

FIG. 1. Silicic acid column fractionation of HFA-treated yeast. Roman numerals show origin of pooled fractions used in fluorine analyses (Table 1).

When fractions representing the four peaks of Fig. 1, were each pooled and analyzed for fluoride, highly positive reactions were obtained from fractions I and II, fraction III showed a weak positive reaction whereas IV was negative. The relative sizes of the reaction spots together with an approximate value for the fluoride they represent are shown in the first line of Table 1. Fraction IV, in which we have been interested would contain citric and fluorocitric acids but these analyses together with many others carried out subsequently failed to show any fluoride in the hydrolyzed samples. Preliminary chromatographic separations of the yeast extracts on paper and developed

in n-butanol–ammonia invariably showed that the acidic spots moved well ahead of authentic citric acid.

Paper chromatographic analyses (see Table 1) revealed the fact that in the sample corresponding to No. I in Fig. 1, two materials were present. These were obtained from the organic phase of the water: 50% chloroform–butanol separation and were readily

TABLE 1. FLUORINE CONTENT OF POOLED FRACTIONS FROM SILICIC ACID COLUMN ANALYSIS OF YEAST EXTRACTS AND CHROMATOGRAPHIC CHARACTERISTICS OF CORRESPONDING FRACTIONS OBTAINED BY DISTRIBUTION OF EXTRACT BETWEEN CHLOROFORM-BUTANOL (I) AND WATER (II)

Analysis	Fraction number			
	I	II	III	IV
Fluorine reactions*	○	○	○	
approximate F content	10 μg	4 μg	trace	—
Paper Chromatography†				
yeast extract: R_f	0·32 0·53	0·087		
Reference acids				
Acetic		0·10 (0·11)‡		
Fluoroacetic		0·096		
Butyric	0·31(0·29)			
Isopentanoic	0·40(0·41)			
n-hexanoic	(0·53)			
n-nonanoic	0·70			
citric	0·00			

* Relative size of reaction spots.[6]
† Ascending; n-butanol:ammonia.[5]
‡ Values in brackets from Block *et al.*.[9]

extracted back into alkaline water without change in the R_f values. Chromatography of samples of authentic n-butyric, iso-pentanoic, n-nonanoic and citric acids gave R_f values, as indicated in Table 1. These mobilities would suggest that the slower moving material in the yeast extract was fluoro-butyric acid, but chromatograms of the only sample of this compound available to us resulted in two spots with R_fs of 0·23, and 0·075. It seemed likely, therefore, that the sample had deteriorated perhaps through lactone formation as suggested by Pattison *et al.*[8] The faster moving spot would appear to have an R_f corresponding to that expected for hexanoic acid.[9]

There is little doubt that the presence of the F atom might alter the mobility of the parent compound, for in higher fatty acids it increases the R_f;[10] but with the exception of HFA, whose mobility is less than acetic acid, the R_fs of the lower fatty acids are unknown. This is no doubt due to the scarcity of authentic samples.

Fraction II, or the aqueous phase from the above separation produced only one spot on the paper chromatogram and when its R_f of 0·087 is compared with those of acetic acid (0·10) and HFA (0·096) its identity as HFA is highly suggestive.

As far as can be ascertained the toxicity tests showed that fraction II was non-toxic, although during the early part of the test the animal exhibited some signs of discomfort. These passed off after 30 min and thereafter no changes in respiration or cardiac

rate and rhythm were detected. The animal receiving an injection in fraction I showed a considerable change in posture and behaviour. Two hours following injection, the cardiac rate had fallen to 156/min from an initial value of 480/min, and arrhythmias were readily detected.

TABLE 2. CITRIC ACID ACCUMULATION IN TISSUES OF RATS INJECTED WITH FRACTIONS OF HFA-TREATED YEAST, COMPARED TO THAT PRODUCED BY INJECTIONS OF SODIUM FLUOROCITRATE AND AN UNFRACTIONATED EXTRACT OF HFA-TREATED YEAST

Tissue	Citric acid μM/gm tissue			
	Chloroform-soluble phase (=Fraction I)	Water-soluble phase (=Fraction II)	Fluorocitrate 8 mg*	Crude yeast extract*
Heart	4·22	4·80	1·37	5·22
Liver	0·81	3·60	9·74	0·16
Kidney	1·98	9·94	23·10	3·81

* Peters (personal communication)

The results of the organ citrate analysis are shown in Table 2, together with the earlier values from the crude (unfractionated) yeast extracts and authentic fluorocitric acid as obtained by Peters. Fluorocitric acid characteristically results in a high concentration of citrate in the kidney, and lower values in the liver, and in the heart. This "pattern" of citrate accumulation was only found in fraction II, and since evidence suggested that it was HFA, the results are consistent with expectations although the absolute values are quite different from fluorocitric acid. Fraction I, the chloroform-soluble fraction, produced quite a different pattern of citric accumulation and resembled most closely that observed in the crude yeast extract. Quite obviously here the greatest accumulation is found in the heart tissue.

DISCUSSION

The identification of the two compounds in Fraction I, as the fluorinated moieties of butyric and hexanoic acids respectively, must at the present time remain tentative. Further work on the identity of these compounds is in progress. That the acids are likely to contain even numbers of carbon atoms is supported by the toxicological data of Pattison *et al.*[8] and Peters,[11] since only such compounds can give rise on breakdown to HFA and thereby toxic effects.[12] Fatty acids are synthesized by the reversal of this catabolic process and therefore only even numbered fluoro-fatty acids should arise from fluoroacetate. Yeasts are known to synthesize lipids from glucose under "resting" conditions[13] and it is likely therefore that in the present investigation the cells would be synthesizing lipid during exposure to HFA.

It is interesting to note that all three of the naturally occurring organic fluorine compounds isolated to date are fluoro-derivatives of alphatic fatty acids, e.g. fluoroacetic in *Dichapetulum cymosium*,[14] —fluorooctadecenoic (fluoro-oleic) and an unidentified acid of higher melting point both in *D. toxicarium*.[15] The fluorine in these compounds must have originally been absorbed from the soil and considering the fact that the fluorine appears in fatty acids, the initial fluoro compound may well have been fluoroacetate.

In contrast to this, animals would appear to synthesize fluorocitrate from fluoroacetate and this observation suggests that there might be a fundamental difference in the fate of the C-2 unit in the plant and animal kingdoms.

On the other hand, it must be remembered that fluoroacetic acid is not the isostere of acetic acid, for the F atom confers physical properties upon the molecule similar to those produced by substitution of OH, CH_3, and NH_2.[16] Thus the isosteres of HFA are glycollic and propionic acids, and glycine and the ultimate fate of HFA in animal or plant cells may depend upon the presence of enzymes concerned with these particular substrates.

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