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The Effect of the γ - and δ -isomers of Hexachlorocyclohexane on the Growth, Fermentation and Respiration of three species of Yeast

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SUMMARY: The growth of three yeast species was inhibited by δ -hexachlorocyclohexane more strongly than by the γ -isomer; the degree of inhibition was related to the inositol requirement of the given yeast. The fermentation of glucose was inhibited only by the δ -isomer and the effect was not reversed by inositol. In one species oxygen uptake was inhibited more strongly by the δ -isomer than by the γ -isomer; only the inhibition caused by the γ -isomer was affected by inositol.

Since Slade (1945) suggested that the toxic effect of γ -hexachlorocyclohexane ('gammexane') might be due to analogue antagonism between this substance and *meso*-inositol a considerable amount of work has been devoted to studying the effect of the isomeric hexachlorocyclohexanes on the growth of a variety of organisms and tissues. Following the demonstration by van Vloten, Kruissink, Strijk & Bijvoet (1948) that it is the δ -isomer whose configuration corresponds to that of *meso*-inositol (hereafter referred to as inositol) the simple theory of antagonism due to stereochemical resemblance had to be abandoned. Although the evidence is somewhat conflicting it seems that both γ - and δ -isomers possess inhibitory or toxic properties for certain biological systems, and that in some cases the inhibition may be partially suppressed by inositol. Kirkwood & Phillips (1946) reported that inositol overcame the effect of gammexane on a strain of yeast for which inositol was an essential nutrient. Fuller, Barratt & Tatum (1950) studied the effect of the γ -isomer on the growth of *Neurospora crassa*, using the wild type and an inositol-requiring mutant. They demonstrated an inhibition which was not annulled by inositol in both strains, and a second type of inhibition annulled by inositol in the mutant strain only. These experiments were criticized by Schopfer (1951) who reported a variable antagonism between the δ -isomer and inositol, quercitol or mytilitol for the inositol-less *Neurospora* when grown in liquid medium. Fuller *et al.* (1950) did not use the δ -isomer; the antagonism between this isomer and inositol has received comparatively little attention. The present work records some observations on the effect of the γ - and δ -isomers of hexachlorocyclohexane on certain aspects of the metabolism of three species of yeast which differ in their requirement for inositol.

MATERIALS AND METHODS

The organisms used were: *Kloeckera apiculata* (*brevis*) (exacting for inositol); *Saccharomyces cerevisiae* Hansen 77 (non-exacting); *S. carlsbergensis* 4228 (intermediate). The culture medium was that of Hopkins & Pennington (1947)

supplemented where necessary with inositol. For the preparation of cell suspensions cultures were grown for 18 hr. (36 hr. in the case of *S. cerevisiae*) at 30° in the liquid medium after which the cells were centrifuged, washed 6 times with 0.02M-KH₂PO₄ (pH 5.6) solution and finally suspended in the same solution diluted as required to obtain a suitable suspension. Since the hexachlorocyclohexanes are very sparingly soluble in water (although somewhat more so in the presence of sugars), stock solutions (0.24 g./100 ml.) were made in ethanol and suitable amounts of these solutions added to the aqueous media.

Growth of the organisms. Portions (9 ml.) of basal medium were placed in 25 ml. flasks and suitable quantities of an ethanol solution of the required isomer added; the total volume was made up to 9.6 ml. with water or inositol solution after which 1 ml. of the standard yeast suspension was added. After incubation at 30° for 18 hr. the crop of cells was removed by centrifugation, uniformly suspended in 25 ml. of 0.02M-phosphate and cell counts carried out by haemocytometer. In each test five replicate experiments were performed.

Carbon dioxide output. CO₂ evolution was measured in the Warburg apparatus at 30°. A suitable yeast suspension in phosphate buffer (1 ml.), together with 0.05 ml. hexachlorocyclohexane solution and 1 ml. water (or inositol solution), was placed in the main compartment of the Warburg flask and 0.6 ml. of 0.1M-glucose in the side tube. After equilibration the glucose solution was run into the main flask and manometer readings taken at 15 min. intervals during periods up to 3 hr.

Oxygen uptake was measured in the Warburg apparatus under similar conditions, except that carbon dioxide was absorbed by KOH placed in the centre cup.

RESULTS

Growth

The results (Figs. 1–3) indicated that the γ - and δ -isomers were to some extent inhibitory to the growth of all three yeasts; the δ -isomer always gave a more marked effect than the γ -isomer for a given concentration. The maximum inhibition was always produced, by both isomers, when the organisms were growing in a deficiency of inositol; even *S. cerevisiae*, considered to be non-exacting, showed a slight response to inositol during an 18 hr. growth period (though not during 30 hr.); in complete absence of inositol it was strongly inhibited by either isomer. This inhibition was, however, almost entirely abolished by 15 μ g. inositol/ml. (Fig. 3). In the presence of an adequate amount of inositol, inhibition by the γ -isomer was greatest for *K. apiculata* and least for *S. cerevisiae*, with *S. carlsbergensis* intermediate; this was in accordance with the respective nutrient requirements of the different organisms for inositol. No such agreement appeared with the δ -isomer for, although *S. cerevisiae* was most strongly inhibited, *S. carlsbergensis* appeared to be more sensitive than *K. apiculata*. Inhibition by the δ -isomer was always less affected by inositol than inhibition produced by the γ -isomer, but in no instance was the inhibition completely overcome by inositol; the degree of

annulment of inhibition reached a certain figure and there remained constant in spite of increased inositol concentrations (see Table 1).

Fig. 1

Fig. 2

Fig. 3

Figs. 1–3. Growth (cell count after 18 hr.) of *K. apiculata*, *S. carlsbergensis* 4228 and *S. cerevisiae* Hansen 77, respectively, in presence of inositol and isomers of hexachlorocyclohexane. Concentrations of isomer: —x—x—, 4.3 $\mu\text{g./ml.}$; —o—o—, 10.8 $\mu\text{g./ml.}$; —□—□—, 21.6 $\mu\text{g./ml.}$; —Δ—Δ—, 43.2 $\mu\text{g./ml.}$; —●—●—, 86.4 $\mu\text{g./ml.}$. Continuous line, γ -isomer; broken line, δ -isomer.

CO₂ output

The γ -isomer had no inhibitory effect on the amount or the rate of CO₂ evolution, and in fact an appreciable increase in the amount evolved in 3 hr. was usually observed (Figs. 4–6). The δ -isomer strongly inhibited fermentation

by all three organisms, and inositol (up to 1 mg./ml.) did not decrease the degree of inhibition. No connexion was evident between the inositol requirement (for growth) and sensitivity to the δ -isomer.

Table 1. *The effect of different concentrations of inositol and the γ - and δ -isomers of hexachlorocyclohexane on growth of certain yeasts*

<i>Kloeckera apiculata</i>				
	Inositol ($\mu\text{g./ml.}$)			
	15	30	45	85
γ -isomer ($\mu\text{g./ml.}$)	Inhibition of growth (% of normal)			
4.3	14	11.5	9	6
10.8	41	30	23	23
21.6	61	54	51	51
43.2	72	68	62.5	63.5
δ -isomer ($\mu\text{g./ml.}$)				
4.3	31	23	17	17
10.8	67	59	52	51
21.6	(100)	(100)	(100)	(100)
<i>Saccharomyces carlsbergensis</i> 4228				
γ -isomer ($\mu\text{g./ml.}$)	0	15	50	95
4.3	29	13	4	3
10.8	54	22	18	18
21.6	81	37	31	29.5
43.2	(100)	65.5	51	46
δ -isomer ($\mu\text{g./ml.}$)				
4.3	54	46	33	31
10.8	(100)	79.5	69	65
21.6	(100)	—	—	(100)
<i>Saccharomyces cerevisiae</i> Hansen 77				
	Inositol ($\mu\text{g./ml.}$)			
	0	15	95	
γ -isomer ($\mu\text{g./ml.}$)	Inhibition of growth (% of normal)			
4.3	9	5.5	4	
10.8	51	11	5.5	
21.6	81	11	5.5	
43.2	93	32	5.5	
δ -isomer ($\mu\text{g./ml.}$)				
4.3	16	8.5	4	
10.8	76.5	29	28	
21.6	92	86	80	
43.2	(100)	(100)	(100)

Fig. 4

Fig. 5

Fig. 6

Figs. 4-6. Carbon dioxide output during 3 hr. by *K. apiculata*, *S. carlsbergensis* 4228 and *S. cerevisiae* Hansen 77, respectively, in presence of hexachlorocyclohexane. Concentrations of isomer: —x—x—, 0 $\mu\text{g./ml.}$ isomer; —●—●—, 45 $\mu\text{g./ml.}$ γ -isomer; —○—○—, 45 $\mu\text{g./ml.}$ δ -isomer; —□—□—, 90 $\mu\text{g./ml.}$ δ -isomer; —△—△—, 135 $\mu\text{g./ml.}$ δ -isomer.

Oxygen uptake

With *S. carlsbergensis* both isomers were inhibitory, the δ -isomer much more than the γ -isomer. A slight decrease of the effect of the γ -isomer was produced by a considerable excess of inositol; under similar conditions no decrease in the inhibition by the δ -isomer was noted (Fig. 7). These experiments were carried out with vigorous young cultures of yeast, and in those experiments concerned with oxygen uptake or CO_2 output the observations were begun not more than 30 min. after the cells were first exposed to the chloro-compounds.

Smith (1951), who investigated oxygen uptake by *S. carlsbergensis*, suggested that the response of the organism to the γ -isomer and to inositol was to some extent dependent on the condition of the yeast culture, i.e. on the age of the culture and the length of time of exposure to the inhibitor. In our hands cultures whose age varied from 15 to 96 hr. showed no differences in response to the γ -isomer as regards growth. The final amount of growth of cultures which had been held at 2° for periods of 1–5 days in the presence of different amounts of the γ -isomer did not differ significantly from controls without the

Fig. 7. Oxygen uptake during 3 hr. by *S. carlsbergensis* 4228 in the presence of inositol and isomers of hexachlorocyclohexane. —x—x—, 0 $\mu\text{g./ml. isomer} + 0 \mu\text{g./ml. inositol}$; —▽—▽—, 135 $\mu\text{g./ml. } \delta\text{-isomer}$; —△—△—, 135 $\mu\text{g./ml. } \delta\text{-isomer} + 350 \mu\text{g./ml. inositol}$; —□—□—, 135 $\mu\text{g./ml. } \gamma\text{-isomer}$; —o—o—, 135 $\mu\text{g./ml. } \gamma\text{-isomer} + 350 \mu\text{g./ml. inositol}$.

γ -isomer. As regards respiration the only notably different response was that of a culture of *S. carlsbergensis* which had been held at 2° for 5 days in the presence of 100 $\mu\text{g. } \delta\text{-isomer/ml.}$ The respiration of this culture was completely inhibited but was appreciably restored by inositol (0.4 mg./ml.). However, examination of cells which had been allowed to stand in contact with the δ -isomer revealed a certain amount of disintegration, and differences in behaviour may have been due to this. In general, in all these experiments the δ -isomer produced a more marked inhibition of respiration than the γ -isomer, and respiration was not significantly restored by excess of inositol.

Note on the solubilities of the isomers of hexachlorocyclohexane. The extremely low solubilities of the isomers in water introduced uncertainty as to the exact concentration of the isomer in solution. In some experiments where the concentration of the γ - or δ -isomer exceeded c. 100 $\mu\text{g./ml.}$, although no precipitate appeared when the medium was prepared some precipitation took place later, largely masked, however, by the yeast cells present. It seemed that the upper limits of true solution in the medium were c. 75 and 100 $\mu\text{g./ml.}$

for the γ - and δ -isomers, respectively. Nevertheless, when 'concentrations' supposedly greater were used there was a further response by the organisms. This may have been due to adsorption of excess of the chloro-compound at the surface of the cells. In any event the quantitative relation between the concentration of chloro-compounds and the response produced cannot be regarded as established until methods for determining true solubility can be applied and it is therefore not possible accurately to compare the inhibitory power of the two isomers except at very low concentrations.

DISCUSSION

It thus appeared that several aspects of the metabolic activities of all three yeasts examined were affected by the γ - and δ -isomers of hexachlorocyclohexane. Cell multiplication was most directly influenced and the effect of the inhibitor was apparently related in some way to the need of an external supply of inositol for the different organisms. The respiratory processes seemed much less sensitive; in all instances the effect of the δ -isomer was much greater than that of the γ -isomer and was much less readily overcome by additional inositol. This is in agreement with the view that the simple theory of analogue antagonism cannot be maintained, and an explanation of the inhibitory effect of both isomers has yet to be found.

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