RESEARCH PAPERS

THE RELATION BETWEEN CHEMICAL STRUCTURE AND UNCOUPLING ACTIVITY IN CONGENERS OF SALICYLATE

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The relation between chemical structure and uncoupling activity in congeners of salicylate has been studied by means of their effects on the oxygen consumption of a suspension of baker's yeast utilising a limited quantity of glucose. Salicylic acid, salicylaldehyde, 2-hydroxyacetophenone, salicylamide, 3-methylsalicylic and 1-hydroxy-2-naphthoic acids were found to show uncoupling activity.

SALICYLATE uncouples oxidative phosphorylation reactions in respiring mitochondrial preparations (Brody, 1956) and many of its effects on the metabolism of isolated tissues and animals are explicable in terms of this action (Smith, 1959). However, the relation between chemical structure and uncoupling activity in the salicylate group of compounds has not been explored in any detail. In the present work, the uncoupling activity of a number of salicylate congeners has been studied by measuring the oxygen consumption of a suspension of starved baker's yeast cells incubated with a known amount of glucose. Uncoupling reagents decrease the proportion of the glucose which is assimilated by the yeast and increase the proportion oxidised, hence stimulating the oxygen consumption of the preparation (Simon, 1953). A preliminary account of the work has already been published (Brostoff, Moses and Smith, 1960).

EXPERIMENTAL

Materials

A 20 per cent (w/v) suspension of baker's yeast (Distillers Co. Ltd.) in 0·067 M KH_2PO_4 solution at pH 4·5 was starved for 16 to 20 hr. at 30°. After centrifugation for 20 min. at about 4500 *g*, the cells were resuspended at the same concentration in a further quantity of the phosphate solution. The salicylate congeners were obtained commercially and recrystallised from suitable solvents until their melting points remained constant. They were dissolved in 0·067 M KH_2PO_4 solution at pH 4·5 to give final concentrations, after admixture with the yeast suspension in the reaction mixtures, ranging from 0·1 to 20 mM.

Total Oxygen Consumption

Aliquots (0·8 ml.) of either the phosphate medium or congener solution were added to Warburg flasks, each of which contained 0·1 ml. of yeast suspension in the main compartment and 0·1 ml. of 0·08 M glucose in the side arm. The flasks were incubated at 32° and the oxygen consumption

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measured for 1 hr. to obtain the initial rate of endogenous respiration of the yeast. The contents of the flask and side arm were then mixed and measurements of the oxygen consumption continued until the phase of stimulated respiration due to the glucose present had ceased and a second rate of endogenous respiration had been established. Typical reaction

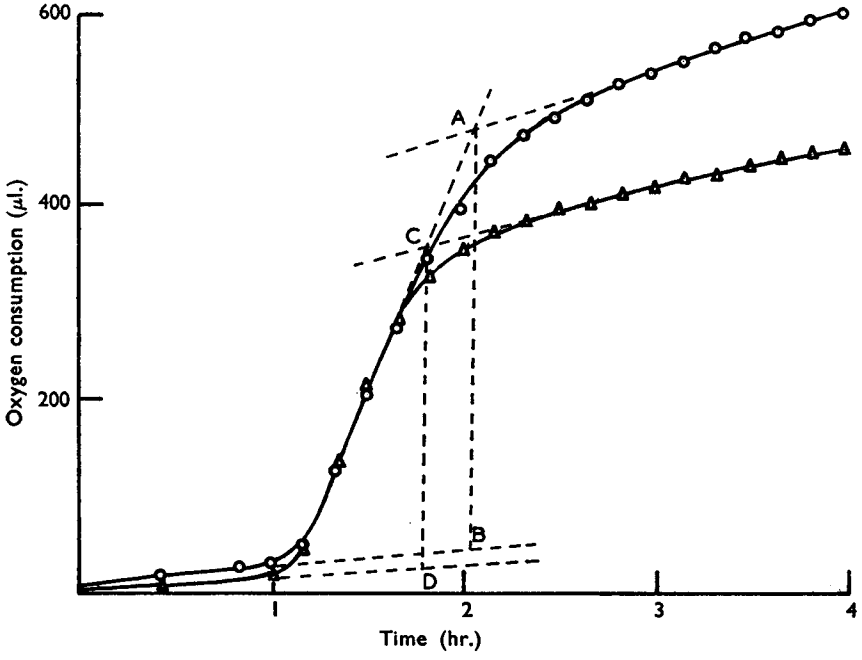

FIG. 1. Oxygen consumption of suspensions of starved baker's yeast cells, in the presence or absence of 10^{-2}M salicylate, before and after the addition of a limited quantity of glucose. Each flask contained 0·1 ml. of yeast suspension (20 per cent w/v of yeast) and 0·8 ml. of congener solution or phosphate buffer in the main compartment. The centre well contained 0·1 ml. of 10 per cent (w/v) KOH, and 0·1 ml. of glucose (8 μmoles) was added to the yeast suspension from the side arm at 1 hr. The total oxygen consumptions resulting from the addition of the glucose were calculated as follows; in the presence of salicylate the difference between the points A and B; control, in the absence of salicylate, the difference between the points C and D. ○, oxygen consumption in the presence of 10^{-2}M salicylate; △, oxygen consumption of the control.

curves are shown in Fig. 1 and the total oxygen consumption resulting from the addition of the glucose was calculated graphically from each experimental curve as the difference between the initial and final levels of endogenous respiration. The result for each concentration of each congener was calculated as the percentage of the corresponding control value obtained in the absence of the congener. A change of 15 per cent was considered to represent a significant effect.

Penetration Experiments

An important consideration in the present work was to determine if the congeners penetrated the cell membranes of the yeast. Although it

was not possible to determine if the active substances reached the enzyme sites concerned with oxidative phosphorylation reactions an attempt was made to assess if the congeners were excluded from the yeast cells. Fresh yeast cells (50 g.) were washed with three successive quantities of 200 ml. of tap water and finally resuspended in 50 ml. of 0·067 M KH_2PO_4 medium at pH 4·5. Aliquots (7·0 ml.) of this suspension were added to stoppered flasks containing 0·5 ml. of 0·01 M glucose and 0·1 to 0·5 ml. quantities of congener solution (0·0063 M in phosphate medium). When necessary the mixtures were made up to a total volume of 8·0 ml. with phosphate medium. The mixtures were shaken mechanically at 32° for 90 min. and centrifuged for 20 min. at about 4,500 *g*. The supernatant solutions were removed and successively frozen and thawed until they were optically clear after centrifugation. The optical densities of these solutions were measured at appropriate wavelengths in 1 cm. cells in a Hilger

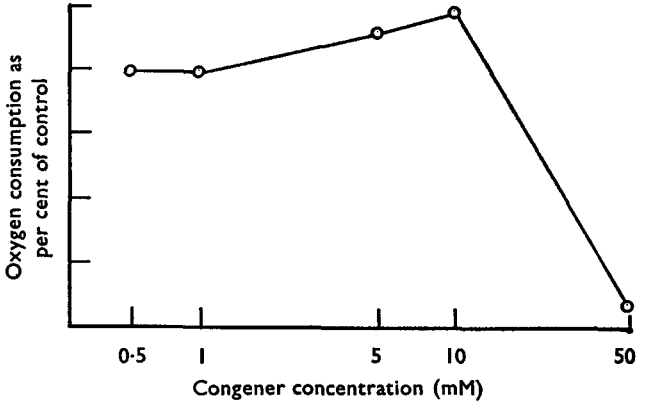

FIG. 2. Stimulation of total oxygen consumption of yeast preparation with increasing concentration of congener followed by depression at highest concentration.

Uvispek spectrophotometer against similar solutions prepared by replacing the congener solution in the reaction mixture with an equivalent quantity of phosphate medium. The salicylate congeners gave sharp absorption maxima in the range 225 to 350 mμ and individual calibration curves were constructed for each substance in phosphate medium. Hexahydrosalicylic acid did not give a suitable absorption maximum and its concentration could not be estimated. The proportion of the total suspension occupied by the yeast cells was found to be 42·5 per cent (v/v) when the suspension was centrifuged until no further packing of the cells occurred. Thus, when 0·5 ml. of 63 $\times$ 10^{-4} M congener solution was present in a total volume of 8·0 ml. of suspension, its concentration in the supernatant after removal of the cells by centrifugation should have been 4 $\times$ 10^{-4} M if it penetrated freely throughout the intracellular volume. A concentration of 7 $\times$ 10^{-4} M of the congener was interpreted as meaning that the congener was completely excluded from the yeast cells. Intermediate results, between 4 and 7 $\times$ 10^{-4} M, indicated partial penetration and values

below 4×10^{-4} M either a preferential binding of the congener by the cells or its chemical alteration.

RESULTS

When the change in total oxygen consumption after the addition of the glucose was plotted against concentration for each congener three types of response were distinguished. The first consisted of a stimulation of the total oxygen consumption with increasing concentration of congener followed by a marked depression at the highest concentration (Fig. 2).

TABLE I

CONCENTRATIONS OF CONGENERS PRESENT IN SUPERNATANT AFTER REMOVAL OF YEAST CELLS AFTER 90 MINUTES INCUBATION*

Congener	Concentration in supernatant (M $\times 10^{-4}$)
Phenol	0
Benzoic acid	1·0
Salicylic acid	0·6
3-Hydroxybenzoic acid	1·0
4-Hydroxybenzoic acid	2·3
2-Methoxybenzoic acid	1·0
Thiosalicylic acid	0·9
Salicylaldehyde	0
2-Hydroxyacetophenone	0
Salicylamide	0·7
Salicylic methyl ester	0
2-Hydroxyphenylacetic acid	6·4
3-Methylsalicylic acid	0
4-Methylsalicylic acid	0·1
3-Phenylsalicylic acid	1·0
1-Hydroxy-2-naphthoic acid	1·3
2,3-Dihydroxybenzoic acid	5·0
2,4-Dihydroxybenzoic acid	9·8
2,5-Dihydroxybenzoic acid	8·7
2,6-Dihydroxybenzoic acid	5·4
3,4-Dihydroxybenzoic acid	8·9
3,5-Dihydroxybenzoic acid	9·1
3-Nitrosalicylic acid	7·5
3,5-Dinitrosalicylic acid	7·4

* Values above 7×10^{-4} M show complete exclusion of congener from the cells, values between 4 and 7×10^{-4} M indicate partial penetration and values below 4×10^{-4} M suggest either preferential binding by the cells or chemical destruction of the congener.

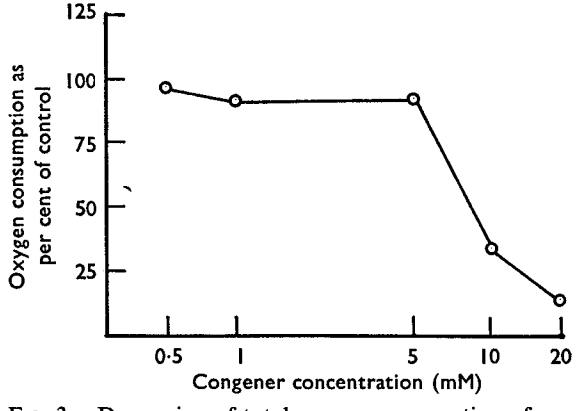

FIG. 3. Depression of total oxygen consumption of yeast preparation with increasing concentration of congener.

The substances which produced this response included salicylate, the classical uncoupling reagent 2,4-dinitrophenol, 3-methylsalicylic acid, 1-hydroxy-2-naphthoic acid, salicylaldehyde, 2-hydroxyacetophenone and salicylamide. All these compounds penetrated the yeast cells (Table I).

The second group of substances, consisting of benzoic acid, 2-methoxybenzoic acid and 3-phenylsalicylic acid, did not cause an initial stimulation of total oxygen consumption with increasing concentration, but produced an increasing depression at the higher concentrations (Fig. 3). All the members of this group were found to penetrate the yeast (Table I). The remaining substances did not cause either stimulation or depression of the total oxygen consumption with increasing concentration (Fig. 4). Some

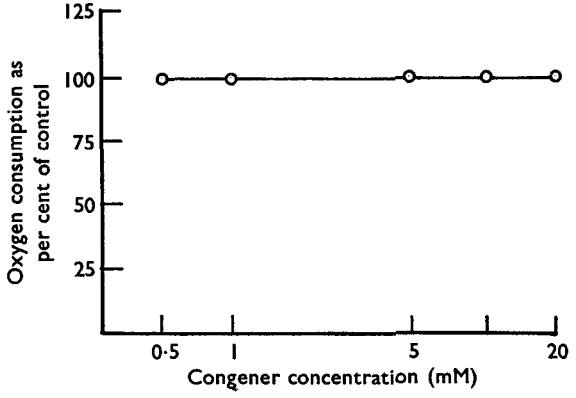

FIG. 4. Unchanged total oxygen consumption of yeast preparation with increasing concentration of congener.

of this last group (3- and 4-hydroxybenzoic acids, 2-hydroxyphenylacetic acid, 2,3- and 2,6-dihydroxybenzoic acids, thiosalicylic acid, 4-methylsalicylic acid, salicylic methyl ester and phenol) were found to penetrate the yeast but the remainder, including 2,4-, 2,5-, 3,4-, and 3,5-dihydroxybenzoic acids, and 3- and 3,5-dinitro-salicylic acids were completely excluded from the cells. *trans*Hexahydrosalicylic acid also gave this type of response but its degree of penetration could not be assessed.

DISCUSSION

When resting cells of baker's yeast are supplied with a limited amount of glucose they oxidise only about 30 per cent of the sugar and the energy produced is used to assimilate the remainder of the glucose into cellular components (Pickett and Clifton, 1943). Simon (1953) has shown that uncoupling reagents, such as the nitrophenols, when present in low concentrations, increase both the rate and total amount of oxygen consumption and reduce the proportion of glucose assimilated by the yeast cells. Thus, a stimulation of the total oxygen consumption of the yeast preparation is a criterion of uncoupling activity. However, this may not occur with all concentrations of the uncoupling reagent since high concentrations of the nitrophenols behave like respiratory poisons, such as

cyanide, in reducing both the oxygen consumption and glucose assimilation (Simon, 1953). In the present work only those substances which, over some portion of the concentration range tested, produced an increase greater than 15 per cent of the total oxygen consumption resulting from the addition of the glucose, were considered to possess uncoupling activity. Of the 25 congeners, only salicylic acid, salicylaldehyde, salicylamide, 3-methylsalicylic acid, 1-hydroxy-2-naphthoic acid and 2-hydroxyacetophenone fulfilled this requirement. The classical uncoupling reagent, 2,4-dinitrophenol, also behaved in the same way. 3-Methylsalicylic acid has been found to increase the oxygen consumption of the whole rat (Andrews, 1958) which presumably indicates an uncoupling activity. Salicylaldehyde (Packer, Austen and Knoblock, 1959) and salicylamide (Brody, 1956) have been reported to be devoid of uncoupling activity in respiring mitochondrial suspensions. The possibility that these two substances may have been almost quantitatively converted to salicylate by the yeast during the incubation was investigated by paper chromatographic analysis of both the incubation media and of extracts of the yeast cells. However, the substances were recovered unchanged, no free salicylate being detected. No information is available about the behaviour of 1-hydroxy-2-naphthoic acid or 2-hydroxyacetophenone in other test systems for uncoupling activity.

Benzoic, 2-methoxybenzoic and 3-phenylsalicylic acids behaved as respiratory depressants in high concentrations but did not cause any stimulation of oxygen consumption over the wide concentration range tested. The remaining congeners possessed neither stimulating nor depressant properties but many of them were completely excluded from the yeast cells. However, 3- and 4-hydroxybenzoic acids, thiosalicylic acid, 4-methylsalicylic acid, salicylic methyl ester, 2,3- and 2,6-dihydroxybenzoic acids, 2-hydroxyphenylacetic acid and phenol penetrated the yeast. In this latter group, the lack of uncoupling ability was therefore due to molecular configuration rather than a failure to penetrate the cellular membranes of the yeast.

The present results show that modification of the hydroxyl group of salicylic acid caused a loss of uncoupling activity. Thus, its absence (benzoic), alteration of its position on the benzene ring (3- and 4-hydroxybenzoic acids), methylation (2-methoxybenzoic) or substitution of the phenolic oxygen by sulphur (thiosalicylic) all produced inactive substances. Alteration of the carboxyl group did not produce such drastic results. The corresponding aldehyde (salicylaldehyde), methyl ketone (2-hydroxyacetophenone) and amide (salicylamide) were active but the methyl ester was not. The absence of the carboxyl group (phenol) or the introduction of a methylene group between the carboxyl and the benzene ring (2-hydroxyphenylacetic acid) also removed activity. Substitution of the benzene ring by a 3-methyl group or by the introduction of a second benzene ring (1-hydroxy-2-naphthoic acid) retained activity but the presence of a 4-methyl, a 3-phenyl group or a second hydroxyl group at the 3 or 6 position produced inactive compounds. All the other ring substituted congeners tested failed to penetrate the yeast. It thus appears that the

essential requirement for uncoupling activity in this group of compounds is the presence of a phenolic hydroxyl group in the *ortho* position to a carboxyl group with the reservation that an aldehyde, ketone or amide group may substitute for the free carboxyl group.

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