activities of chloroplasts isolated in non-aqueous media (Stocking, 1959), and which have not been leached of some water-soluble components. But the suggested role of glycollic oxidase in the respiration of illuminated leaves (Zelitch, 1959) does not necessarily conflict with glycollic oxidase occurring outside chloroplasts; although glycollic acid is formed in chloroplasts, unlike some other products of photosynthesis it diffuses readily into the surrounding cytoplasm (Tolbert, 1959).

SUMMARY

1. Preparations from tobacco leaves were centrifuged in sucrose density gradients and resolved into chloroplasts, chloroplast fragments and particles, presumably mitochondria, containing succinoxidase activity.

2. The centrifuging sedimented fumarase as if it were attached to the mitochondria.

3. Cytochrome oxidase sedimented as if it were associated with mitochondria, and some mitochondrial fragments. No activity was detected in chloroplasts either before or after disruption with digitonin.

4. Glycollic oxidase did not sediment appreciably during the centrifuging. It could not be detected in intact chloroplasts.

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The Effect of Sulphate Assimilation on the Induction of Arylsulphatase Synthesis in Fungi

BY T. HARADA AND B. SPENCER
Institute of Scientific and Industrial Research, Osaka University, Japan, and Department of Biochemistry, Trinity College, Dublin

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Arylsulphatases of various types are present in most animals, including mammals, birds, amphibia and molluscs (Dodgson & Spencer, 1956). In bacteria the enzyme is not widely distributed but it has been found in strains of *Aerobacter aerogenes* (Harada, 1952), *Salmonella*, mycobacteria (Whitehead, Morrison & Young, 1952), *Streptococcus alcalophilus* (Harada, Kono & Yagi, 1954), *Alcaligenes metalcaligenes* (Dodgson, Melville, Spencer & Williams, 1954) and in other isolated species. In fungi, the arylsulphatase of Taka-diastase (Neuberg & Korono, 1923) and other commercial preparations (Abbot, 1947) of *Aspergillus oryzae* has long been known and the presence of the enzyme in other fungi has been noted (Harada *et al.* 1954).

The only sulphate ester known to be present in fungi, apart from the sulphatophosphates, adenosine 5′-sulphatophosphate and adenosine 3′-phosphate 5′-sulphatophosphate (Spencer & Harada, 1960), is choline sulphate which appears to be restricted to higher fungi (Harada & Spencer, 1960). It was possible that choline sulphate or the sulphate–phosphate anhydrides could act as endogenous inducers of arylsulphatase synthesis and to

investigate this possibility an attempt was made to correlate choline sulphate synthesis and arylsulphatase activity in a number of fungi.

No such relationship was observed but during the investigation significant differences in arylsulphatase levels were noticed depending on the sulphur source used in the culture medium in which the fungi were grown. This phenomenon is discussed in terms of diauxie (Monod, 1941) concerning arylsulphatase synthesis and the assimilation of sulphur compounds.

EXPERIMENTAL

Fungi. Initial strains were supplied by the Commonwealth Mycological Institute except *Rhizopus formosaensis*, IFO 4776, and *Mucor javanicus*, IFO 4569, which were obtained from the Institute of Fermentation, Japan.

Culture conditions. Fungi were grown at 30° in shaking culture in 5 or 100 ml. of media, which were inoculated with 1 drop or 0·5 ml. respectively of a spore suspension containing 10^7–10^8 spores/ml. in sterile distilled water. The chemically defined medium contained per litre: glucose, 10 g.; asparagine, 1·5 g.; $(NH_4)_2HPO_4$, 1·5 g.; KH_2PO_4, 1·0 g.; $MgCl_2,4H_2O$, 0·5 g.; NaCl, 0·5 g.; inositol, 20 mg.; thiamine, 1 mg.; biotin, 1 μg. and the appropriate amount of sulphur-containing compound (equivalent to 65 mg. of S). Media were adjusted to pH 6·6 before autoclaving. Sulphite, thiosulphate, sulphide, cysteine-sulphonate and sulphate esters, apart from choline sulphate which is stable during sterilization by autoclaving, were Seitz-filtered, after adjustment of their respective solutions to pH 6·6, and then added to the sterilized medium.

Preparation of acetone-dried mycelia. Mycelia were harvested by filtering through muslin and washed well with water and excess of water was removed. The mycelia were ground in a cooled mortar with acetone at 0° and left in cold acetone for 1 hr. The acetone was then quickly removed by filtration under suction and the precipitates were washed well with cold acetone. The mycelia were finally dried *in vacuo* over P_2O_5. *Nematospora gossypii*, baker's yeast, *Saccharomyces cerevisiae*, *Saccharomyces cerevisiae* var. *ellipsoideus*, *Pichia membranaefaciens*, *Sporobolomyces salmonicolar*, *Torulopsis utilis* and *Rhodotorula rubre* were harvested and acetone-dried by centrifuging.

Estimation of p-*nitrophenol produced in culture media.* To 5 ml. of culture medium was added potassium *p*-nitrophenyl sulphate to give a final concentration of 1 mM (equivalent to 0·16 mg. of S/5 ml.) and, in some cases, another sulphur source equivalent to 0·325 mg. of S/5ml. Fungi were grown for 3 days at 30° with shaking. Growth was stopped and the yellow colour of liberated *p*-nitrophenol developed by addition of 1 ml. of 0·5N-NaOH. After filtering or centrifuging, the *p*-nitrophenol in the medium was measured colorimetrically with the Kotaki AKA colorimeter and filter S-43 (maximum transmission at 435 mμ, band width 395–485 mμ). A standard curve prepared with 0–120 μg. of *p*-nitrophenol/5 ml. sample was linear. Control determinations, to correct for non-specific production of yellow-coloured substances during growth of the fungi, were carried out with culture media to which potassium *p*-nitrophenyl sulphate had not been added.

Determination of arylsulphatase activity. To tubes containing a suspension of 10 mg. of acetone-dried mycelium in 2 ml. of water was added 1 ml. of 0·5M-acetate buffer, pH 6·3, and the mixtures were preincubated at 34° for 5 min. The substrate, 1 ml. of 0·01M-potassium *p*-nitrophenyl sulphate, was added, and after incubation at 34° for 20 min. or 1 hr., depending on the activity present, 1 ml. of 0·5N-NaOH was added and *p*-nitrophenol measured as before. Control determinations were carried out in which the mycelium suspension and substrate were incubated separately and combined immediately before the addition of NaOH.

RESULTS

Hydrolysis of p-*nitrophenyl sulphate by growing fungi.* A convenient and rapid way of surveying a number of fungi for the occurrence of arylsulphatase was to use *p*-nitrophenyl sulphate as the sole sulphur source for fungal growth. Alkali was added after a suitable period of growth and arylsulphatase activity was shown by the yellow colour of liberated *p*-nitrophenol. With this method, all but three of the fungi tested (group 4, Table 1) appeared to produce the enzyme.

The addition of other sulphur sources to the culture medium had a pronounced effect on the liberation of *p*-nitrophenol. The activity of the group 1 fungi (Table 1) was either abolished or markedly diminished when inorganic sulphate or cysteine were the additional sulphur sources; the arylsulphatase activities of group 2 fungi were affected by inorganic sulphate but hardly, or not at all, by cysteine; the group 3 fungi were unaffected by either cysteine or sulphate. When choline sulphate or taurine were the additional sulphur sources the arylsulphatase activities of all groups of fungi were the same as, or greater than, when *p*-nitrophenyl sulphate alone was the sulphur source. Only with one or two fungi did these two additional compounds bring about any diminution in arylsulphatase activity (Table 1).

The decrease in rate of hydrolysis of *p*-nitrophenyl sulphate when certain fungi were grown in the presence of cysteine or inorganic sulphate seemed unlikely to have been caused by direct inhibition of arylsulphatase activity since, at the concentration used in the culture media (approximately 2 mM), neither of these two sulphur compounds inhibited the arylsulphatase activity *in vitro* of the acetone-dried mycelia of *Mucor hiemalis* and *Ustilago scitaminia*. Nor could the decrease in enzyme activity be due to a general inhibition of fungal growth, since for any one fungus the mycelial dry weights did not vary greatly with the sulphur source used in addition to *p*-nitrophenyl sulphate. Growth was rather less when *p*-nitrophenyl sulphate was the sole sulphur source and with *Sacch. cerevisiae*, *Sacch. cerevisiae* var. *ellipsoideus* and *Sporobolomyces salmonicolar* only

Table 1. *Hydrolysis of* p*-nitrophenyl sulphate by fungi growing on culture media containing various sulphur supplements*

Fungi were grown in 5 ml. of the standard medium supplemented with a sulphur compound (0·325 mg. of S/5 ml.) and *p*-nitrophenyl sulphate (NPS) (0·16 mg. of S/5 ml.) where indicated. After 3 days' growth at 30° with shaking the *p*-nitrophenol liberated was measured.

Strain	*p*-Nitrophenol liberated (μg./5 ml. of culture medium)				
	NPS	NPS + K_2SO_4	NPS + choline sulphate	NPS + taurine	NPS + cysteine
Group 1					
Mucor hiemalis (−) CMI 21217	300	0	420	420	0
M. javanicus IFO 4569	280	0	260	270	33
Cunninghamella echinulata CMI 44534 (a)	68	0	71	68	0
Baker's yeast	340	0	350	390	60
Aspergillus terreus LSHTM	410	60	650	660	95
Penicillium aurantio-brunneum LSHTM P2	410	26	400	420	80
Group 2					
Nematospora gossypii CMI 31279	12	0	10	15	8
Neurospora crassa CMI 19419	450	53	480	510	220
N. sitophila (+) CMI 63919	370	0	390	550	400
Ustilago scitaminia CMI 63920	370	0	510	500	430
Aspergillus oryzae CMI 17299	360	40	360	360	150
Asp. sydowi CMI 63904	650	100	600	650	550
Penicillium citreo-roseum LSHTM P22	360	40	170	120	170
P. chrysogenum CMI 62728	310	0	300	360	390
Rhodotorula rubre CMI 38784	170	33	80	100	200
Fusarium sp.	170	0	12	150	85
Group 3					
Rhizopus stolonifer (+) CMI 57761	510	500	650	650	500
Rhi. formosaensis IFO 4776	390	160	350	440	390
Pichia membranaefaciens NCYC 57	450	320	230	220	280
Aspergillus nidulans CMI 16643	330	170	310	450	430
Penicillium notatum LSHTM P45	510	430	300	300	360
Oedocephalum glomerulosum CMI 60070	270	100	640	480	85
Torulopsis utilis CMI 23311	550	450	510	630	500
Group 4					
Saccharomyces cerevisiae CMI 19391	0	0	0	0	0
S. cerevisiae var. *ellipsoideus* NCYC 57	0	0	0	0	0
Sporobolomyces salmonicolar CMI 56578	0	0	0	0	0

slight growth was observed on this medium. No release of *p*-nitrophenol corresponding to this slight growth was observed. However, similar slight growth occurred with these fungi when there was no added sulphur source in the medium, and the growth can therefore be attributed to the presence of small sulphur reserves in these organisms or to sulphur compounds introduced in the inoculum or as trace impurities in the media. *Sporobolomyces* grew well on the supplemented media, but whereas both strains of *Saccharomyces* grew well when SO_4^{2-} ions and cysteine were the sulphur supplements growth was poor with taurine and choline sulphate.

In these culture experiments it was possible that the *p*-nitrophenol liberated from the substrate by the action of arylsulphatase might have been metabolized to colourless products. Fresh rat-liver homogenates are known to give false arylsulphatase values when *p*-nitrophenyl sulphate is the substrate, owing to the reduction of the liberated *p*-nitrophenol to *p*-aminophenol (Dodgson & Spencer, 1953). Recoveries of *p*-nitrophenol from cultures of growing fungi were checked by incorporating 70 μg. of *p*-nitrophenol (Seitz-filtered) to 5 ml. of the synthetic medium containing SO_4^{2-} ions. After 3 days' growth in shaking culture the amount of *p*-nitrophenol remaining was measured. With 16 of 24 tested strains recoveries of *p*-nitrophenol were complete (95–100 %). Some *p*-nitrophenol disappeared with three strains, *Rhizopus stolonifer* 15 μg. loss, *Cunninghamella echinulata* 43 μg., *Penicillium chrysogenum* 26 μg., suggesting that some reduction of the nitro group or complete catabolism of the *p*-nitrophenol had occurred. The extinction (S-43 filter) of the culture media of the remaining strains increased, *Neurospora sitophila* 15 % increase, *U. scitaminia* 60 %, *Sporobolomyces salmonicolar* 10 %, *Aspergillus nidulans* 10 %, and the colour developed at pH 10 was orange. These

Table 2. *Arylsulphatase activity of acetone-dried mycelia of fungi grown on different sulphur sources*

Sulphur sources	Arylsulphatase activity (μg. of *p*-nitrophenol produced/hr./10 mg. of acetone-dried powder)			
	Mucor hiemalis	*Aspergillus nidulans*	*Rhizopus stolonifer*	*Ustilago scitaminia*
K_2SO_4	0	16	240	0
Na_2SO_3	0	0	130	0
$Na_2S_2O_3$	0	11	260	0
L-Sodium cysteinesulphonate	24	30	1300	0
L-Cysteine–HCl	0	34	380	44
L-Methionine	0	36	140	68
Glutathione	53	58	190	150
Na_2S	42	130	1600	94
Taurine	72	210	1600	250
Cysteic acid	210	150	1700	420
Cysteinesulphinic acid	110	160	1300	430
Choline sulphate	140	37	1500	320
Phenyl sulphate	97	30	280	12
p-Nitrophenyl sulphate	170	18	160	13
Phenolphthalein disulphate	120	170	650	45

increases could have been due either to oxidation of *p*-nitrophenol to more highly chromogenic di- or tri-hydroxynitrophenols or to the production of natural products absorbing in the region of 400 mμ. In the experiments reported in Table 1 allowance was made for the latter eventuality by using a control in which potassium *p*-nitrophenyl sulphate had been omitted. Accurate data could not be obtained with *T. utilis* because of the production of excessive amounts of substances absorbing light at the wavelength of measurement of *p*-nitrophenol.

Arylsulphatase activity in acetone-dried fungi. In order to obtain a more precise knowledge of the dependence of arylsulphatase synthesis in fungi on the sulphur source used for growth of the mycelia, fungi were grown on a standard medium to which was added different sources of sulphur. The mycelia were collected, acetone-dried, and the arylsulphatase activity measured. The major part of the arylsulphatase produced by the fungi resided in the mycelia. When *Asp. nidulans* or *Rhiz. stolonifer* were cultured in media in which inorganic sulphate or taurine was the sulphur source, less than 10% of the total arylsulphatase activity was present in the culture filtrate.

The arylsulphatase activities of acetone-dried mycelia of representative fungi from group 1, *M. hiemalis*, group 2, *U. scitaminia*, and group 3, *Rhiz. stolonifer* and *Asp. nidulans*, are shown in Table 2. For *Ustilago* grown on SO_4^{2-}, SO_3^{2-}, $S_2O_3^{2-}$ ions and cysteinesulphonate and for *Mucor* grown on SO_4^{2-}, SO_3^{2-}, $S_2O_3^{2-}$ ions, cysteine and methionine, no arylsulphatase activity could be detected. Both the *Aspergillus* and *Rhizopus* produced relatively small amounts of the enzyme when grown on SO_4^{2-}, SO_3^{2-}, $S_2O_3^{2-}$ ions, cysteine and methionine as compared with the mycelia grown on other sulphur sources. In general, the mycelia of all the fungi exhibited high activity when grown on taurine, cysteate, cysteinesulphinate or S^{2-} ions and, in most cases, choline sulphate and arylsulphates produced high enzyme levels. Mycelia grown on cysteinesulphonate contained relatively high activities for *Rhizopus* and *Mucor*, whereas strains of *Aspergillus* and *Ustilago* developed little or no activity. These differences will be discussed.

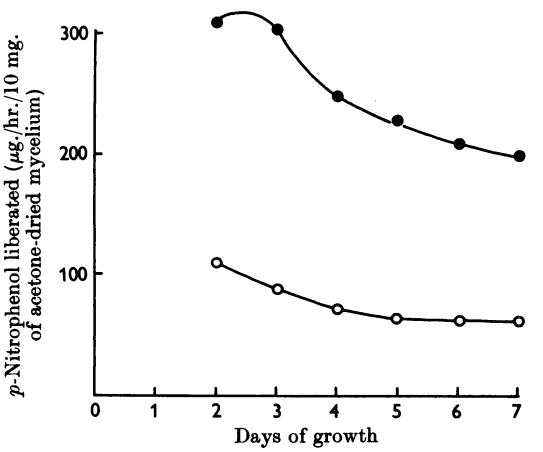


Fig. 1. Arylsulphatase activity in mycelia after growth for different periods in taurine medium. ●, *Ustilago scitaminia*; ○, *Mucor hiemalis*.

The arylsulphatase activities reported in Table 2 were those of mycelia harvested after 4 days' growth. By harvesting *Ustilago* and *Mucor* after different periods of growth on SO_4^{2-} ions or taurine medium it was seen (Fig. 1) that maximum activity, expressed on the basis of mycelial dry weight, occurred after 2 days and thereafter it declined.

Table 3. *Arylsulphatase activity of acetone-dried mycelia grown at different* pH *values*

Arylsulphatase activity is expressed as μg. of *p*-nitrophenol liberated/hr./10 mg. of acetone-dried mycelium.

Sulphur source in culture medium	Initial pH	Arylsulphatase activity	Final pH of medium	Arylsulphatase activity	Final pH of medium
K_2SO_4	4·8	0	5·0	0	3·2
	6·0	0	5·2	0	3·3
	7·6	0	6·6	0	5·9
Taurine	4·8	23	5·2	83	3·1
	6·0	35	5·4	120	3·2
	7·6	60	7·2	150	5·5

Table 4. *Occurrence of arylsulphatase*

Fungi were grown on glucose (1 %, w/v)–peptone (1 %, w/v)–yeast-extract (0·5 %, w/v) medium or the standard medium incorporating taurine as the sulphur source. The mycelia were acetone-dried and the arylsulphatase activity was measured. The data for choline sulphate production is from Harada & Spencer (1960).

	Arylsulphatase activity (μg. of *p*-nitrophenol produced/hr./10 mg. of acetone-dried mycelium)		
Phycomycetes	Glucose–peptone–yeast-extract medium	Taurine medium	Choline sulphate production
Mucorales			
Rhizopus stolonifer (+) CMI 57761	160	1600	–
Rhi. formosaensis IFO 4776	0	150	–
Mucor hiemalis (–) CMI 21217	0	72	–
M. javanicus IFO 4569	0	3	–
Cunninghamella echinulata CMI 44534 (a)	0	50	–
Ascomycetes			
Endomycetales			
Nematospora gossypii CMI 31279	0	0	–
Saccharomyces cerevisiae CMI 19391	0	0	–
S. cerevisiae var. *ellipsoideus* NCYC 57	0	0	–
Baker's yeast	0	5	–
Pichia membranaefaciens NCYC 51	0	78	–
Sphaeriales			
Neurospora crassa CMI 53238	0	3	+
N. sitophila (+) CMI 63919	0	830	+
Basidiomycetes			
Ustilaginales			
Ustilago scitaminia CMI 35616	0	250	+
Fungi imperfecti			
Moniliales			
Sporobolomycetaceae			
Sporobolomyces salmonicolar CMI 56578	0	0	+
Moniliaceae			
Aspergillus terreus LSHTM Ab2	0	480	+
Asp. oryzae CMI 17299	6	910	+
Asp. sydowi CMI 63904	0	18	+
Asp. nidulans CMI 16643	59	210	+
Penicillium citreo-roseum LSHTM P22	0	38	+
P. chrysogenum CMI 62728	0	48	+
P. notatum LSHTM P45	0	270	+
P. aurantio-brunneum LSHTM P2	0	8	+
Oedocephalum glomerulosum CMI 60070	0	66	+
Trichoderma viride sp.	0	12	+
Pseudosaccharomycetaceae			
Torulopsis utilis CMI 23311	0	40	–
Rhodotorula rubre CMI 38784	0	2	+
Tuberculariaceae			
Fusarium sp.	0	520	+

During the period 2–7 days, arylsulphatase activity declined by 47 (*Mucor*) and 27 % (*Ustilago*) while the total mycelial weight increased by about 50 and 35 %, respectively. The decrease in enzyme activity on a dry-weight basis may thus be principally due to a dilution effect, enzyme synthesis stopping after 2 or 3 days whereas mycelial growth continued. No arylsulphatase activity was observed in either *Mucor* or *Ustilago* grown on SO_4^{2-} ions at any period up to 7 days.

In the experiments summarized in Table 2, the final pH of the culture medium varied slightly, depending upon the sulphur compound used. Thus with *Rhiz. stolonifer* the final pH varied between 4·0 and 5·2; *M. hiemalis*, 5·0 and 5·7; *Asp. nidulans*, 4·0 and 5·2; *U. scitaminia*, 3·5 and 4·0. This pH variation does not appear to be the reason for the absolute differences in arylsulphatase activity brought about by the use of different sulphur sources, since SO_4^{2-} ions failed to produce arylsulphatase activity in *M. hiemalis* or *U. scitaminia* over a wide pH range (Table 3) whereas the activity with taurine as the sulphur source was high at all pH values.

It appeared from the results in Tables 1 and 2 that if a fungus was a potential producer of arylsulphatase, enzyme activity would not be detected if SO_4^{2-} ions or cysteine were the sulphur sources used for its culture; on the other hand, the use of taurine would allow the synthesis of the enzyme. The failure to produce arylsulphatase was also seen to extend to a glucose–peptone–yeast-extract medium since, of the acetone-dried mycelia of 27 strains tested, only *Rhiz. stolonifer*, *Asp. oryzae*, and *Asp. nidulans* showed activity (Table 4). With the synthetic medium incorporating taurine as the sulphur source all but four strains produced arylsulphatase. Three of these inactive strains, *Nematospora gossypii*, *Sacch. cerevisiae* and *Sacch. cerevisiae* var. *ellipsoideus*, belong to the Endomycetales; the other, *Sporobolomyces salmonicolar*, is a member of the Moniliales. With *Sporobolomyces salmonicolar* and *Nematospora gossypii* taurine was effectively used as a sulphur source for growth but both strains of *Saccharomyces* showed only weak growth on the taurine medium.

Arylsulphatase activity in Aspergillus nidulans *mutants.* A number of cysteine-less mutants of *Asp. nidulans*, originally prepared by Hockenhull (1949), were grown on the synthetic medium with taurine or cysteine as the sulphur source. The arylsulphatase activity of the acetone-dried mycelia of each of the three mutants, *gamma*, *iota* and *eta*, which could utilize sulphite and taurine but not sulphate, was greater on the taurine medium than on the cysteine medium. The other mutants, *alpha*, *beta* and *zeta*, could not utilize sulphate, sulphite or taurine. When grown on the cysteine medium the acetone-dried mycelia of these mutants had an arylsulphatase activity of the same order as that of the *gamma*, *iota* and *eta* mutants grown on cysteine rather than taurine (Table 5).

Table 5. *Arylsulphatase activity of acetone-dried mycelia of* Aspergillus nidulans *mutants grown on media containing cysteine and taurine as sulphur sources*

Mutants	Arylsulphatase activity (μg. of *p*-nitrophenol liberated/hr./10 mg. of acetone-dried mycelium)	
	Taurine medium	Cysteine medium
Alpha	No growth	132
Beta	No growth	68
Gamma	402	93
Iota	276	66
Eta	612	150
Zeta	No growth	80

DISCUSSION

There is sufficient difference between the occurrence of choline sulphate and arylsulphatase synthesis in fungi to suggest that the sulphate ester does not act as an obligatory endogenous inducer of the enzyme. All of the strains of Phycomycetes produced arylsulphatase (Table 4) but none produced choline sulphate (Harada & Spencer, 1960). In Endomycetales, two out of five strains produced the enzyme but none produced the sulphate ester.

One strain of Sporobolomycetaceae failed to show arylsulphatase activity but synthesized choline sulphate. The other 15 strains, belonging to the Sphaeriales in the Ascomycetes, the Basidiomycetes or Fungi imperfecti, were arylsulphatase and choline sulphate positive except that *T. utilis* was arylsulphatase positive and choline sulphate negative. Of the *Asp. nidulans* mutants, *iota* and *gamma* were unable to synthesize choline sulphate due to blocks in the synthesis of adenosine 3′-phosphate 5′-sulphatophosphate (unpublished results) but all produced arylsulphatase even on the cysteine medium.

A further possibility, that adenosine 5′-sulphatophosphate or adenosine 3′-phosphate 5′-sulphatophosphate act as endogenous inducers of arylsulphatase, seems ruled out because the anhydrides are obligatory intermediates in choline sulphate synthesis. The evidence from the *Asp. nidulans* mutants and the fact that the two strains of *Saccharomyces* can both produce these anhydrides but fail to show arylsulphatase synthesis emphasize this point.

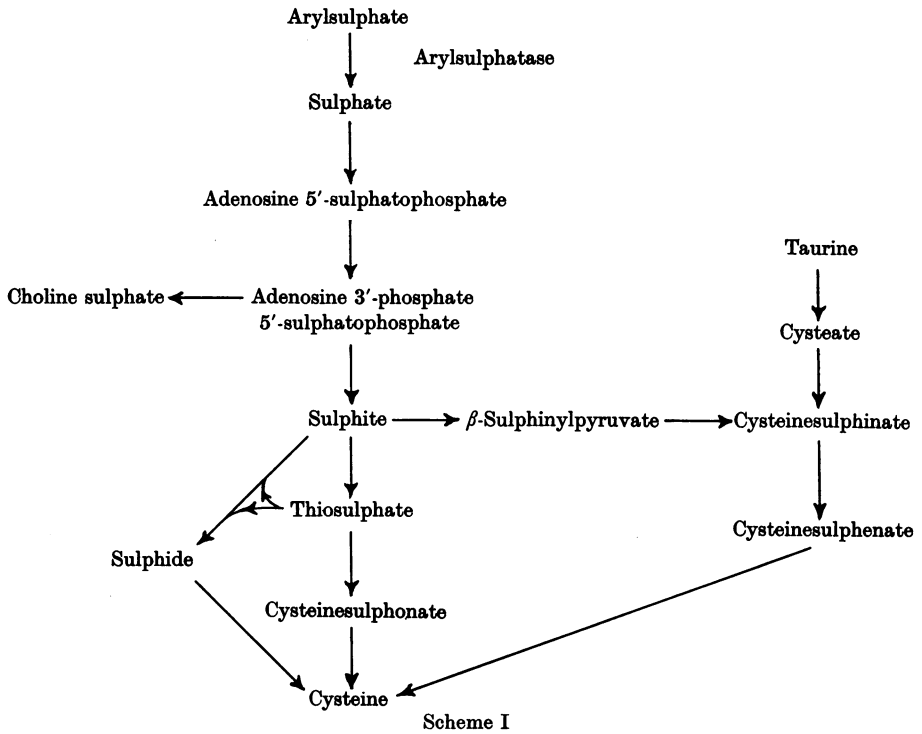
The variety of sulphur compound used as the sole source of sulphur in the culture medium

markedly affected arylsulphatase production (Table 2). Some correlations can be made between these effects and the role of the sulphur compounds in the assimilation of inorganic sulphate to cysteine. It seems clear that the primary stage in the assimilation of sulphate by micro-organisms is a reduction to sulphite via adenosine 5′-sulphatophosphate and adenosine 3′-phosphate 5′-sulphatophosphate (Wilson & Bandurski, 1958; Hilz & Kittler, 1958; Hilz, Kittler & Knape, 1959; Bandurski, Wilson & Asahi; 1960; Peck, 1960). Three pathways have been proposed for the further assimilation of SO_3^{2-} ions to cysteine as shown in Scheme I.

Studies on growth requirements of cysteine-less mutants of *Asp. nidulans* have suggested that thiosulphate and cysteinesulphonate are intermediates (Hockenhull, 1949; Nakamura & Sato, 1960). Similar studies on *Asp. nidulans* mutants by Shepherd (1956) led to the formulation of a scheme for sulphite utilization via β-sulphinylpyruvic acid and cysteinesulphinic acid. Growth studies on *Escherichia coli* mutants (Cowie, Bolton & Sands, 1950) and enzyme studies on yeast (Hilz *et al.* 1959; Wainwright, 1961; Schlossman & Lynen, 1957) suggested a pathway in which sulphide is an intermediate. In all of the proposed schemes cysteate and taurine are not considered to be direct intermediates in the assimilation of inorganic sulphate to cysteine and it is significant that with these sulphur sources relatively large amounts of arylsulphatase developed in all the tested strains (Table 2) whereas direct intermediates, such as SO_4^{2-}, SO_3^{2-} and $S_2O_3^{2-}$ and cysteine, produced only weak activity or none at all.

Fundamentally, the question to be decided in attempting to explain these findings is whether taurine, cysteate, choline sulphate, etc. are inducers of arylsulphatase, whereas SO_4^{2-}, SO_3^{2-}, $S_2O_3^{2-}$ ions etc. are not, or whether the former compounds allow arylsulphatase synthesis and that the latter compounds are actually repressors of the synthesis of the enzyme. The second suggestion is more in keeping with current thought on the mechanism of enzyme induction and is supported by the evidence that when the fungi listed in Table 2 are grown on a medium containing both taurine and inorganic sulphate the arylsulphatase level in the mycelia is zero or the same as that when SO_4^{2-} ions are used as the sole sulphur source.

The fact that inorganic sulphate is a product of arylsulphatase activity and that it repressed the synthesis of the enzyme in the four fungi listed in Table 2 suggests that it is a direct repressor of arylsulphate synthesis. Repression of the induction of enzymes by the product of the enzyme action is already familiar with tryptophan (Monod & Cohen-Bazire, 1953), methionine (Cohn, Cohen & Monod, 1953) and arginine (Gorini & Maas, 1957) synthetases whose induction is inhibited by the



Scheme I

specific amino acids that they produce. A more pertinent example is the inhibition of phosphatase induction in *E. coli* by inorganic phosphate (Horiuchi, Horiuchi & Mizuni, 1959; Torriani, 1960).

If it is assumed that inorganic sulphate is a direct repressor, two hypotheses can be put forward to explain the findings shown in Table 2. Both hypotheses invoke the 'diauxie' phenomenon which is familiar in the utilization of carbon and nitrogen sources by bacteria and in the inhibition of β-galactosidase induction (Monod, 1941; Magasanik, 1955; Harada, 1959; Cohn & Horibata, 1959). Current theory considers that the large number of structurally unrelated compounds that can inhibit the induction of a specific enzyme are metabolized to a common repressor. In the present case it is known that fungi can metabolize almost any sulphur source to inorganic sulphate by various oxidative mechanisms, which are not necessarily the reverse reactions of the sulphate assimilation pathway. In the first of the hypotheses the different repressor activities of the various sulphur compounds would be explained by different rates of oxidation to inorganic sulphate. Thus sulphite is probably quickly oxidized to sulphate whereas taurine, cysteate and choline sulphate are used primarily for growth purposes and are only slowly oxidized to sulphate in repressor amounts. This would explain why the maximum arylsulphatase activity of fungi grown on taurine is after 2 days' growth and thereafter it declines (Fig. 1). The second hypothesis invokes a negative feed-back mechanism along the sulphate utilization pathway. With sulphur sources that are direct intermediates in sulphate assimilation the assimilation pathway below the specific intermediate would be blocked by negative feed-back and any SO_4^{2-} ions formed by side-path oxidation would thus accumulate in sufficient amount to repress arylsulphatase synthesis. The SO_4^{2-} ions formed from compounds like taurine and cysteate, which are not on the direct pathway of sulphate utilization, would be assimilated quickly and would only accumulate in repressor amounts when sufficient free cysteine had been formed from the taurine or the cysteate to exert negative feed-back along the sulphate assimilation pathway. By an extension of this latter hypothesis it would appear that since cysteinesulphinic acid produces high arylsulphatase levels with all the fungi it cannot be a direct intermediate between sulphite and cysteine and indeed no evidence for this pathway, other than mutant-growth experiments, has been adduced. Also (see Scheme I) both β-sulphinylpyruvic acid and cysteinesulphenic acid are only hypothetical compounds. Other results quoted in Table 2 would suggest that *Asp. nidulans* and *U. scitaminia* favour the pathway via cysteinesulphonate rather than that via sulphide. The lower fungi *Rhiz. stolonifer* and *M. hiemalis* produce high arylsulphatase levels when grown on sulphide, cysteinesulphinate and cysteinesulphonate and it would have to be argued that the pathway of sulphate utilization by these organisms is via a route other than those shown in the Scheme.

SUMMARY

1. The liberation of *p*-nitrophenol from potassium *p*-nitrophenyl sulphate by certain fungi during growth is inhibited by the addition of cysteine or inorganic sulphate to the culture medium, whereas taurine and choline sulphate have little effect.

2. The arylsulphatase activity of acetone-dried powders of the mycelia of *Mucor hiemalis*, *Aspergillus nidulans*, *Rhizopus stolonifer* and *Ustilago scitaminia* varied with the sulphur sources used in the culture medium. In general, sulphate, sulphite, thiosulphate, cysteine and methionine gave low arylsulphatase levels whereas taurine, cysteic acid, cysteinesulphinic acid and choline sulphate gave high levels. The response to cysteinesulphonic acid and sulphide varied with each fungus.

3. In a survey of the acetone-dried mycelia of 27 fungi grown on a glucose–peptone–yeast-extract medium only *Rhizopus stolonifer*, *Aspergillus oryzae* and *Aspergillus nidulans* showed arylsulphatase activity. In a chemically defined medium in which taurine was the sole sulphur source all but four of the fungi, *Nematospora gossypii*, *Saccharomyces cerevisiae*, *Saccharomyces cerevisiae* var. *ellipsoideus* and *Sporobolomyces salmonicolar*, showed arylsulphatase activity.

4. These findings have been interpreted as a 'diauxie' effect in which the inhibitors of arylsulphatase synthesis are metabolized to a common repressor which may be inorganic sulphate.

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Studies on the Biosynthesis of Starch Granules

2. THE PROPERTIES OF THE COMPONENTS OF STARCHES FROM SMOOTH- AND WRINKLED-SEEDED PEAS DURING GROWTH*

BY C. T. GREENWOOD AND J. THOMSON
Department of Chemistry, The University, Edinburgh 9

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Mature pea starches have been reported to contain more amylose than the 20–25% usually present in root or cereal starches, there being about 35% in smooth-seeded and about 70% in wrinkled-seeded varieties (McCready, Guggolz, Silveira & Owens, 1950). Work on these starches by Potter, Silveira, McCready & Owens (1953) indicated that, although the amylose components were similar, the amylopectin component from a wrinkled-seeded variety had an abnormal length of unit-chain of 36 glucose units and a smaller molecular size than that from a smooth-seeded pea. These hitherto unconfirmed results suggest that either some radical alteration has occurred in the balance of the synthesizing enzymes, or a new type of branch-synthesizing enzyme exists.

In the work described here the components from mature smooth- and wrinkled-seeded pea starches have been characterized by measurements of extents of conversion by β-amylase, iodine absorption, periodate oxidation and molecular size. These properties have been compared with those of typical root and cereal starches. Further, in an attempt to gain some insight into the method of biosynthesis of the starch granules, the properties of pea starch and its component amylose and amylopectin during growth have been studied.

* Banks & Greenwood (1959) is regarded as Part 1.

EXPERIMENTAL

Isolation of starches. Freshly gathered peas were exhaustively extracted with 0·01 M-$HgCl_2$ and then deproteinized as described by Banks & Greenwood (1959). (It has been found that the starch in dried peas can be similarly extracted and purified if the peas are initially soaked overnight in 0·01 M-$HgCl_2$ at room temperature.) The purified starches were then defatted with boiling aqueous 80% methanol. The varieties of pea investigated and the properties of the isolated starches are given in Table 1.

Development of granules. Peas were harvested at various stages of growth and graded in size by sieves before the starch was extracted as above.

Table 2 shows the properties of the resultant materials. Percentages of starch were estimated by the method described by MacWilliam, Hall & Harris (1956). Gelatinization temperatures were measured as described by Banks & Greenwood (1959).

Fractionation of starches. Fractionation was achieved by both conventional dispersion and aqueous-leaching experiments (Banks & Greenwood, 1959; Banks, Greenwood & Thomson, 1959). In agreement with the work of Potter *et al.* (1953), pea starches would not disperse directly into water and pretreatment with liquid NH_3 was necessary. This technique causes no modification of the molecular size or β-amylolysis limit of amylose (Banks *et al.* 1959).

The purity of the separated components was measured as described by Banks & Greenwood (1959). β-Amylolysis limits were measured with purified soya-bean β-amylase