albumin in these conditions whereas the others cannot.

Whether or not the presence of salt is necessary to start the combination of two proteins, it certainly greatly increases it. As in causing flocculation, the efficiency of different salts in influencing complex formation depends on the valency of the ion with the charge opposite to that of the protein particles. The two processes, however, are not identical. If the protein is denatured by heating in the absence of salts, the subsequent addition of salt causes flocculation. By contrast, salts influence complex formation only if they are present in protein mixtures during heating, for the addition of salt to a protein mixture heated salt-free does not lead to formation of any measurable amount of serologically non-precipitating complexes. This difference could be explained by assuming that individual proteins differ in their rates of aggregation, and that these rates are much greater than rates of denaturation. Then, if salt is present during heating, particles of different proteins would be able to combine with one another as soon as they are denatured, but if salt is added to a mixture of proteins already denatured by heating in salt-free solution, each protein would tend to aggregate separately.

The results with antibodies almost exactly parallel those with antigens. The two antisera used were to bushy-stunt virus and to human-serum albumin. When heated in the presence of salt, these behave similarly, the antibodies forming non-precipitating complexes which inhibit precipitation. In the absence of salts the two antisera behaved differently. The virus antiserum again ceased to precipitate and instead inhibited precipitation, though less strongly than when heated with salt, whereas the antiserum to human albumin still precipitated. This difference at

first sight suggests differences in the heat stabilities of the two antisera. A similar difference between the behaviour of antisera to 'O' and 'H'-type antigens is well known and was for long interpreted as evidence that these antigens produced antibodies with different properties. It has now been shown, however, that antibodies to antigens of both types undergo similar changes when heated, and that the difference in their behaviour occurs because the precipitability of the antigens is affected differently by the presence of antibodies changed into non-precipitating complexes [Kleczkowski, 1941*b*; Bawden & Kleczkowski, 1941, 1942]. Similarly, the difference between the behaviour of dialysed antisera to human albumin and to bushy-stunt virus respectively, may be because the two antigens are affected differently by the presence of non-precipitating antibody complexes, which are probably formed to a small extent when both antisera are heated in the absence of salts.

SUMMARY

The formation of complexes between different proteins, undergoing heat denaturation together, is greatly influenced by the presence of salts. In the absence of salts only mixtures containing water-soluble serum globulin formed any detectable amount of complexes.

The efficiency of different salts in promoting the complex formation, expressed as the reciprocal of the lowest effective concentration, follows Hardy's law. Ions of higher valency are much more effective than those of lower valency. On the alkaline side of the isoelectric point of the proteins, the efficiency of salts is determined by the valency of the cations, and on the acid side by the valency of anions.

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The Influence of Halide Concentration on the Metabolism of *Penicillium sclerotiorum* van Beyma

By D. REILLY AND T. P. CURTIN, *The National University of Ireland (University College, Cork)*

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In a previous communication [Curtin & Reilly, 1940], the isolation and properties of sclerotiorine, a chlorine-containing metabolic product of *Penicillium sclerotiorum* van Beyma, were described.

Reference was also made to a second product, a red pigment retained by the mother-liquors of crystallization. Work on the constitution of sclerotiorine was somewhat hampered by the small yield, 2% of

the dry mycelium weight. The possibilities of increasing this figure by alteration of the chloride concentration and by activation with yeast extract [Abraham, Chain, Fletcher, Florey, Gardner, Heatley & Jennings, 1941] have now been investigated, the basal medium being that of Czapek-Dox. Simultaneously, the organism has been grown on media devoid of chlorides, viz. Raulin-Thom and Raulin-Dierckx media together with a Czapek-Dox medium compounded without KCl, in the hope of obtaining the red component in a more workable form. The mycelium pigmented in all cases, and the dark red product of light petroleum extraction was submitted to diverse processes to free it from the fats and other products of the extraction. Sublimation in a high vacuum, separation by adsorption on Al_2O_3 and $\text{Ca}(\text{OH})_2$, and crystallizations at the interface of two immiscible liquids gave no substance that could be isolated other than as a glaze on glass.

Inoculations of the mould on Czapek-Dox media, containing bromides or iodides in amounts equivalent to the usual KCl concentration, gave no crystalline bromo- or iodo-derivatives. This finds a parallel in the work of Raistrick & Smith [1936]. However, appreciable darkening in the colour of the mycelium and its extract was noted with each change of halide.

Cultural conditions. The culture used throughout was the 6th transfer from that obtained in March 1940 from the Centraalbureau voor Schimmelcultures, Holland. Sucrose was used as the source of carbon, trial cultures proving that this sugar was satisfactory.

The substrate employed was the acid Czapek-Dox medium (sucrose, 50 g.; NaNO_3 , 2 g.; KH_2PO_4 , 1 g.; KCl, modified; MgSO_4 , $7\text{H}_2\text{O}$, 0.5 g.; FeSO_4 , $7\text{H}_2\text{O}$, 0.01 g.; distilled water to 1000 ml.). Four batches, each of 5 l., containing 0.25, 0.5, 0.75 and 1.0 g. KCl/l., respectively as the sole source of chlorine were prepared. The media were distributed in 100 ml. lots in 20 oz. flat bottles, the cotton-wool plugs being paper-capped to prevent dilution of the medium during sterilization by steaming on each of three consecutive days. The bottles were inoculated with a spore suspension from a 14-day-old culture of *P. sclerotiorum* on malt agar, and incubated at 25°. Three bottles were filtered at the pump every 3 days, and the filtrate tested for pH, sugar concentration by polarimeter and for residual KCl.

In all inoculations described, the pH value reached a minimum on the 9th day and reverted to approximate neutrality on the 24th day, in agreement with the figures already published [Curtin & Reilly, 1940]. The production of sclerotiorine was thus uninfluenced by the acidity of the medium. The polarimeter readings were of particular interest. Due to their similarity, one series will suffice as an illustration (see Table 1).

Table 1

Days of incubation	Polarimeter reading
0	5.7° D
3	2.6° D
6	0.3° L
9	1.0° L
12	0.6° L
15	0.0
18	0.0
21	0.0
24	0.0

The connexion between the minimum pH value on the 9th day and the maximum *laevo* reading is obvious. The main interest centres round the fact that more than four-fifths of the sucrose was metabolized in so short a period as 9 days.

Estimation of residual chloride. Direct titration was not possible as the medium reduces both KMnO_4 and AgNO_3 . Oxidation by refluxing with conc. HNO_3 was unsatisfactory. A modified form of the method described by Raistrick & Smith [1936] was finally employed:

50 ml. of the filtered medium were shaken for 10 min. with 2.5 g. of norit charcoal and filtered at the pump without washing. 25 ml. of this filtrate were taken and excess AgNO_3 added, together with 5 ml. of 6N HNO_3 , which had been boiled free from HNO_2 . The precipitated AgCl was coagulated by boiling, filtered off and washed with a little dilute HNO_3 . On cooling below 25°, the new filtrate was titrated against N/100 NH_4CNS , using 1 ml. of standard Volhard indicator.

In actual practice, the medium extracts chlorides from the norit, the KCl equivalent, as determined by a series of blanks, being 0.00095 g./25 ml. of the medium. The residual percentage KCl, at intervals, over a period of 24 days is shown in Table 2.

Table 2

Days of incubation	Initial chloride content of media			
	0.25 g. KCl/l.	0.5 g. KCl/l.	0.75 g. KCl/l.	1.0 g. KCl/l.
	Residual chloride content			
	%	%	%	%
0	0.0250	0.0497	0.0747	0.0994
3	0.0172	0.0369	0.0527	0.0762
6	0.0071	0.0274	0.0509	0.0735
9	0.0050	0.0258	0.0499	0.0729
12	0.0070 (?)	0.0250	0.0473	0.0705
15	0.0033	0.0250	0.0470	0.0678
18	0.0027	0.0235	0.0437	0.0661
21	0.0015	0.0233	0.0457 (?)	0.0640
24	0.0009	0.0230	0.0475 (?)	0.0640
Metabolized KCl	0.0241 %	0.0267 %	0.0310 %	0.0354 %

Plotting the residual KCl against time gives four curves of identical shape, which show that the utilization of KCl becomes regular after 9 days. Before this, the halide is rapidly taken up. We have

already stressed the rapidity with which the sugar is utilized during this same period.

The filtered mycelium from 25 bottles (2.5 l.) of each of the four media was pressed, vacuum-dried, and extracted with light petroleum. A mean value of 12.9 g. dry mycelium/l. was taken as the basis of calculation. The extracted sclerotiorine was weighed on recrystallization, the yields of pigment being practically constant, allowing for the necessarily incomplete separation from the solvent (see Table 3, col. 2).

Completion of the chlorine balance sheet was effected by the estimation, as KCl, of the chloride content of the extracted mycelium. The method [Schiff, 1879; 1906] was as follows:

Mycelium (2-3 g.) was weighed into a small platinum crucible and covered with an intimate mixture of 1 part pure Na_2CO_3 and 4 parts pure CaO and inverted into a larger crucible which was then completely filled with the same mixture. The whole was then heated with a blow-pipe so that the outer parts attained a high temperature before the mycelium began to decompose. After heating to redness and cooling, the mixture was treated with distilled water, the solution being acidified with pure HNO_3 , an appreciable rise in temperature being avoided. The solution was then adjusted to known volume and the chlorides estimated by the Volhard method already described.

The metabolized KCl and the KCl equivalents of sclerotiorine and the mycelia are given in Table 3. The 5th column, KCl unaccounted for, shows the quantities of KCl retained by the mother-liquors of crystallization, its presence having been duly verified.

Table 3

KCl/l. of medium g.	Yield of sclero- tiorine g./l. medium	KCl meta- bolized as pigment g./100 ml. medium	KCl as mycelium %	KCl un- accounted for %
0.25	0.190	0.0038	0.0127	0.0076
0.50	0.213	0.0042	0.0118	0.0107
0.75	0.189	0.0038	0.0128	0.0144
1.00	0.220	0.0044	0.0136	0.0174

The results so far may be summarized as follows: Yields of pigment and the chlorine content of the mycelium are, within the limits of experimental error, practically constant, while the chlorine content of the mother-liquors are in direct proportion to the KCl concentration of the medium.

Effect of pH and addition of yeast extract. A further series of inoculations to test the influence of pH and yeast extract on the rate of KCl metabolism was now completed, pH, polarimeter and Volhard titrations being performed as already described. For the influence of pH, 5 l. of neutral Czapek-Dox medium were made up by K_2HPO_4 instead of KH_2PO_4 as in the acid medium (pH 4.2).

Yeast extract was prepared by macerating 500 g. pressed yeast with 1 % soda solution, re-pressing on the Buchner funnel and washing with distilled water. The yeast, suspended in 1 l. of distilled water, was boiled for 1 hr. and allowed to stand overnight, when it was centrifuged and the volume of the clarified extract adjusted to 1000 ml. The extract was then sterilized by steaming on three consecutive days. Two batches of 5 l. standard Czapek-Dox medium were then made up, incorporating 5 and 10 % by volume respectively, of the yeast-water sown and incubated as above. All three media showed the same rapid elimination of sugar, but the pH of the neutral medium, on reaching its minimum value, displayed only a slight tendency to revert to neutrality. In estimating the KCl content of the yeast-extract media, it was found necessary to add an additional blank of 0.00044 g. for each 5 % of yeast water. The corrected KCl percentages are shown in Table 4.

Table 4

Days of incubation	Media		
	Neutral Czapek-Dox	Neutral Czapek-Dox plus 5 % yeast extract	Neutral Czapek-Dox plus 10 % yeast extract
	% residual KCl content of media (corrected)		
0	0.0497	0.0500	0.0496
3	0.0375	0.0434	0.0482
6	0.0354	0.0422	0.0452
9	0.0339	0.0405	0.0434
12	0.0315	0.0400	0.0425
15	0.0309	0.0350	0.0375
18	0.0309	0.0200	0.0202
21	0.0295	0.0154	0.0200
24	0.0250	0.0140	0.0173
Metabolized KCl	0.0247 %	0.0360 %	0.0323 %

The disappearance of KCl in the neutral medium is slower though more regular than in the acid medium. The yeast water decelerates the initial KCl metabolism up to the 12th day, after which the halide content drops rapidly until the 15th day. It seems reasonable therefore to assume that the activating effect of yeast extract is first directed towards the sugar metabolism to the detriment of the chloride, but that when the sugar has been eliminated, the activation is completely centred round the utilization of the KCl present.

The decreased rate of KCl metabolism during the early stages of growth definitely reduces the yield of pigment. In all three of the above inoculations, the amounts of recrystallized sclerotiorine obtained were only slightly in excess of 1 % of the dry mycelium weight.

SUMMARY

Growth of *P. sclerotiorum* on media containing KBr and KI in place of the usual KCl did not yield halide derivatives analogous to sclerotiorine, the ordinary

chlorinated metabolic product, while growth on media devoid of chloride in no way assisted the isolation of the red component. Variation of the KCl concentration showed that its elimination from the medium was quite regular after the initial period when the sugar metabolism was exceptionally rapid. Increased chloride concentration had no effect on the production of sclerotiorine and only affected the chlorine content of the other products of extraction

of the mycelium. It is suggested that the pigment is a metabolic by-product rather than the fundamental end-product of the chloride metabolism. The activating effects of yeast extract was no more than equivalent to the effect of increased KCl concentration.

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The Determination of 2:3-Diketo-*l*-gulonic Acid

BY J. R. PENNEY AND S. S. ZILVA (*Member of the Scientific Staff, Medical Research Council, The Division of Nutrition, Lister Institute, London*)

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The first stage of the oxidation of *l*-ascorbic acid is of particular interest to the biochemist. The product, dehydro-*l*-ascorbic acid, retains its lactone ring and can be reduced to *l*-ascorbic acid by mild reagents such as H_2S , etc., in vitro, and can also be regenerated in vivo almost quantitatively. In aqueous solutions, dehydro-*l*-ascorbic acid undergoes mutarotation which is generally assumed to be due to the gradual opening of its lactone ring with the formation of 2:3-diketo-*l*-gulonic acid. This latter compound can still be converted into *l*-ascorbic acid by lactonization and reduction when treated with HI in vitro [Herbert, Hirst, Percival, Reynolds & Smith, 1933]. Regeneration apparently does not occur in vivo, since little if any antiscorbutic activity has been observed in solutions of dehydro-*l*-ascorbic acid which have undergone mutarotation for some time [Hirst & Zilva, 1933; Borsook, Davenport, Jeffreys & Warner, 1937].

Whilst dehydroascorbic acid can easily be determined by titration of the regenerated ascorbic acid after reduction with H_2S , no direct method is available for the determination of diketogulonic acid. In this communication conditions are described under which this compound, which so far has not been isolated, can be determined directly.

Herbert *et al.* [1933] have shown that both ascorbic acid and freshly oxidized ascorbic acid give derivatives with 2:4-dinitrophenylhydrazine of m.p. 282° (decomp.) and 280° (decomp.) respectively. We have found that diketogulonic acid obtained by mutarotation from dehydroascorbic acid also gives with 2:4-dinitrophenylhydrazine a red derivative or derivatives which on solution in NaOH produce an intensely red colour. This colour reaction forms the foundation on which the procedure for the direct determination of the diketogulonic acid is based.

2:4-Dinitrophenylhydrazine reacts also with phenylpyruvic acid [Penrose & Quastel, 1937] and with other α -keto acids [Lu, 1939; Sealock & Silberstein, 1940; Sealock & Scherp, 1941], and consequently the reaction cannot be considered specific. In actual practice, however, it has been found that extracts of the tissues of normal guinea-pigs, and untreated human urine give a very low and fairly constant colour with dinitrophenylhydrazine; thus it should be possible to detect the appearance of even small quantities of diketogulonic acid by this reaction. Furthermore, the results suggest that the procedure outlined by us is open to further improve-