above agents in the necrotic destruction of tissues with a simultaneous enhancement of the NAD-glycohydrolase activity.

SUMMARY

1. The optimum conditions for the heat-activation of the enzyme nicotinamide-adenine dinucleotide glycohydrolase present in an inhibited state in the crude cell-free extracts of the virulent strain of *Mycobacterium tuberculosis* H₃₇R_v have been described.

2. The heat-activated enzyme was partially purified by ammonium sulphate fractionation, iso-electric precipitation, and calcium phosphate-gel adsorption.

3. The optimum conditions of temperature, pH, substrate and enzyme concentrations are presented, and the K_m values for both the substrates, namely NAD and NADP, are given. The enzyme differs from the NAD glycohydrolases reported from other micro-organisms in having a sharp pH optimum and equal reactivity towards NAD and NADP.

4. The effect of various inhibitors and anti-tubercular agents on the partially purified enzyme has been studied.

5. The inhibitor with which the enzyme is associated in the crude cell-free extracts appears to be specific for the enzyme from this source. However, the inhibitor from the avirulent strain *M. tuberculosis* H₃₇R_a was inhibitory for the partially purified enzyme from the virulent strain to an equal extent.

6. Evidence for the presence of an excess of free inhibitor in the crude cell-free extracts is presented and the complex formed between the enzyme and its inhibitor is shown to be undissociable.

REFERENCES

- Abdel Latif, A. A. & Alivisatos, S. G. A. (1962). *J. biol. Chem.* **237**, 500.
 Alivisatos, S. G. A. (1959). *Nature, Lond.*, **183**, 1034.
 Alivisatos, S. G. A. & Woolley, D. W. (1956). *J. biol. Chem.* **219**, 823.
 Artman, M. & Bekierkunst, A. (1961). *Proc. Soc. exp. Biol., N.Y.*, **106**, 610.
 Bekierkunst, A. & Artman, M. (1962). *Amer. Rev. resp. Dis.* **86**, 832.
 Chaudhuri, S. N., Suter, E., Shah, N. S. & Martin, S. P. (1963). *J. exp. Med.* **117**, 71.
 Colowick, S. P. (1955). In *Methods in Enzymology*, vol. 1, p. 97. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
 Goldman, D. S. (1954). *J. Amer. chem. Soc.* **76**, 2841.
 Gopinathan, K. P., Sirsi, M. & Ramakrishnan, T. (1963). *Biochem. J.* **87**, 444.
 Green, S. & Bodansky, O. (1962). *J. biol. Chem.* **237**, 1752.
 Kaplan, N. O., Colowick, S. P. & Nason, A. (1951). *J. biol. Chem.* **191**, 473.
 Kern, M. & Natale, R. (1958). *J. biol. Chem.* **231**, 41.
 Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
 Narrod, S. A., Bonavita, V., Ehrenfeld, E. R. & Kaplan, N. O. (1961). *J. biol. Chem.* **236**, 931.
 Rajalakshmi, S., Sarma, D. S. R. & Sarma, P. S. (1963). *Indian J. exp. Biol.* **1**, 63.
 Scaife, J. F. (1963). *Canad. J. Biochem. Physiol.* **41**, 1469.
 Stirpe, F. & Aldridge, W. N. (1961). *Biochem. J.* **80**, 481.
 Windmueller, H. G. & Kaplan, N. O. (1962). *Biochim. biophys. Acta*, **56**, 388.
 Youmans, G. P. & Karlson, A. G. (1947). *Amer. Rev. Tuberc.* **55**, 529.
 Zatman, L. J., Kaplan, N. O. & Colowick, S. P. (1953). *J. biol. Chem.* **200**, 197.
 Zatman, L. J., Kaplan, N. O., Colowick, S. P. & Ciotti, M. M. (1954). *J. biol. Chem.* **209**, 453.

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Regulation of Enzyme Synthesis in an Enucleate Cell

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A currently popular theory proposes that the regulation of enzyme synthesis in the cell is controlled by the flow of a special type of short-lived RNA from the gene to the site at which the enzyme is synthesized (Monod & Jacob, 1961; Brenner, Jacob & Meselson, 1961; Gros *et al.* 1961). It has been suggested that the amount of enzyme formed bears some relationship to the amount of RNA

synthesized on the gene concerned (Hiatt, Gros & Jacob, 1963); and since it is supposed that this RNA is rapidly destroyed after transfer to the cytoplasm, its continued production is held to be necessary for continued synthesis of the enzyme.

The evidence in support of this theory is indirect and rests heavily on experiments carried out on micro-organisms, especially *Escherichia coli*. How-

ever, the theory can be tested in a more direct way in certain animal and higher-plant cells which survive enucleation, at least insofar as the residual cytoplasm remains capable of protein synthesis. The fact that protein is synthesized in the cytoplasm of amoebae and of human amnion cells for several hours after enucleation (Prescott, 1959, 1960; Goldstein, Micou & Crocker, 1960), and in the cytoplasm of the giant unicellular alga *Acetabularia* for many weeks (Hämmerling, Clauss, Keck, Richter & Werz, 1959), makes it obvious that the synthesis of protein in these cells does not require the continued synthesis of short-lived RNA molecules in the nucleus. But no attempt appears to have been made to examine whether the synthesis of a specific enzyme can be regulated in the enucleate cell.

In many respects *Acetabularia* is the most suitable organism for such an investigation since this cell is capable not only of protein synthesis but of specific morphogenetic development in the absence of the nucleus (Hämmerling, 1953). The present study deals with three phosphatases found in *Acetabularia crenulata*. The synthesis of these enzymes was studied during the period of regeneration which follows removal of the cap in these cells. Since this regeneration can occur in the absence of the nucleus a comparison was made between the nucleate and the enucleate cell. One of the phosphatases is specifically associated with the development of the cap, and it is synthesized in large amounts only when the cap is being formed. It was found that the synthesis of this enzyme took place in the normal way when the cap was reformed, after amputation, in the enucleate cell. This finding indicates not only that a specific enzyme can be synthesized in the absence of the nucleus, but that this synthesis can be regulated in the enucleate cell.

MATERIALS AND METHODS

Conditions of growth of A. crenulata. The cells were grown from cysts obtained from Professor J. Hämmerling of the Max-Planck Institut für Meeresbiologie, Wilhelmshaven, Germany. The conditions of culture were those described by Beth (1953), and the medium was the *Erdschreiber* described by Hämmerling (1944). The sea water was obtained from the Fladen ground and soil with a low clay content was used. The temperature of the growth rooms was maintained at 21–22° and the cells were illuminated 12 hr. daily by Mazda 3500° K white fluorescent lamps at an intensity of 2200 lux. Swarming of the gametes took place at 23–25°, the cells being illuminated for 8.5 hr. daily. The medium was changed at 3-weekly intervals and the cell density in the cultures progressively reduced to maintain optimum growth. Under these conditions the generation time of the cells was 8–9 months. The cultures were fertile.

Enucleation and removal of the cap. The nucleus of *Acetabularia* lies within the rhizoid at the base of the stalk. The cell can therefore be enucleated simply by cutting off

the rhizoid. This is done with de Wecker scissors (John Weiss and Son Ltd., London). The cap can also be conveniently removed with these scissors. The amputation is done in a dish of *Erdschreiber* medium and the stalk is not moved for 2–3 min. afterwards to permit the cytoplasm at the cut edge to collapse away from the cell wall and thus seal the central vacuole from the sea water. In some cases there is a generalized contraction of the cytoplasm. All cells from which parts have been removed are kept in the dark overnight; during this period the cytoplasm returns to its original state. This transient contraction of the cytoplasm appears to have no subsequent effect on the growth of the cell.

It has been shown that, if the cap and the rhizoid are removed simultaneously, regeneration of the cap occurs only rarely (Hämmerling, 1943). However, a high proportion of enucleate cells will re-form caps if the cells are kept in the dark for a few days between removal of the caps and removal of the rhizoids (Hämmerling, 1943). In the present experiments where cap regeneration was to be studied after enucleation the cells from which the caps had been removed were kept in the dark for 8 days before removal of the rhizoids. After these had been removed the cells were kept in the dark for a further day to allow them to recover from the operation.

Assay of phosphatase activity. The following buffers were used. They were prepared with 0.4N reagents, the final concentration being 0.2N in the first component. (1) pH 5.0, acetic acid–potassium hydroxide. (2) pH 7.7, tris-hydrochloric acid. (3) pH 8.5, tris-hydrochloric acid. (4) pH 12.0, glycine–potassium hydroxide.

Each assay required about 30 mature cells or up to 300 immature cells. These were washed in water and then homogenized in a glass Potter–Elvehjem homogenizer in 2 ml. of tris buffer, pH 7.7. The insoluble material was spun off and the supernatant assayed.

Acid-phosphatase activity was measured by a modification of the method of Boman (1955) with *p*-nitrophenyl phosphate as substrate. The pH optimum of the acid-phosphatase activity in *A. crenulata* was found to be 5.0. The assay was therefore carried out in buffer of this pH at 37° with 1 mM substrate, which gave a maximal rate of reaction. The pH of the reaction mixture was 5.0. Samples of the reaction mixture were taken at 5 min. intervals and pipetted into equal volumes of 0.5N-potassium hydroxide. The extinction of the samples was read at 400 m μ . Under these conditions the release of *p*-nitrophenol was linear over an incubation period of 20–30 min. The rate of the reaction was calculated from the first 20 min.

Alkaline-phosphatase activity was measured by a modification of the method of Garen & Levinthal (1960), again with *p*-nitrophenyl phosphate as substrate. Alkaline-phosphatase activity in *A. crenulata* was found to have two pH optima: 8.5 and 12.0. Assays were therefore carried out in buffers of these pH values with 1 mM substrate, which gave a maximal rate of reaction. The pH of the reaction mixtures was 8.4 and 11.7. The release of *p*-nitrophenol was measured directly at 400 m μ in a spectrophotometer with a temperature-controlled cell-holder maintained at 37°. Under these conditions the release of *p*-nitrophenol was linear over a period 20–30 min., and the rate of the reaction was calculated from the first 20 min.

Magnesium was assayed at various concentrations, but was not found to have any activating effect. Zinc had a

slight inhibitory effect. Metal ions were therefore not added to the reaction mixtures.

In the present experiments all the results are expressed in units of enzyme activity per cell. A unit of enzyme activity is defined as the activity which releases $1 \mu\text{mole}$ of *p*-nitrophenol/min. at 37° in the presence of excess of substrate.

The enzyme activity at all three pH optima exhibited typical Michaelis-Menten kinetics. The enzyme activity was lost when the solution was heated at 90° or incubated with trypsin or chymotrypsin at 37° .

At pH 12 a slow non-enzymic hydrolysis of *p*-nitrophenyl phosphate takes place. The rate of this hydrolysis was therefore subtracted from the experimental values obtained at this pH to give the rate of enzymic hydrolysis.

RESULTS

Synthesis of phosphatases during normal cell growth. The increases in the activities of the three phosphatases during normal cell growth are shown in Fig. 1. Since cells of the same chronological age may have reached different stages of development, the enzyme activity is related to cell length. This is an acceptable measure of the stage of development because in any one set of cultural conditions there is little variation in the length of the cells at the time of cap formation. In the cultures used for the present experiments caps were formed when the cells were 33–38 mm. long. It will be seen that the activities of all three enzymes increased during the growth of the cell; but there was a marked increase

in the activity of the pH 12 enzyme, relative to the other two, at the time of formation of the cap.

Synthesis of phosphatases in the regenerating nucleate cell. In *Acetabularia*, formation of the cap is normally followed by repeated division of the nucleus and migration of the secondary nuclei from the rhizoid into cysts within the cap. If the cap is removed before nuclear division occurs, this division is delayed until another cap is formed. The cap may be removed repeatedly at this stage and, over a period of several years, each removal is followed by regeneration of a new cap (Hämmerling, 1963).

In the present experiments the caps were removed when the cells were 35 mm. long and a period of 3–4 weeks elapsed between removal of the cap and the reappearance of the whorls which herald the formation of a new cap. The whole process of regeneration of the cap took 6–8 weeks. The cells were studied over a period of 70 days, during which time there was little increase in their length. The new caps attained a normal size. Samples of 50 cells were harvested at 10-day intervals for each set of assays. A few of the cells did not show any signs of regeneration and these were discarded from the sample. The activities of the three phosphatases during the course of the experiment are shown in Fig. 2. It will be seen that re-formation of the cap

Fig. 1. Increases in the activities of the three phosphatases during normal cell growth. The generation time of the cells was about 8 months. Whorls appeared about 6 months after formation of the zygotes, and caps were mature about 2 months later. $\circ$, pH 5 enzyme; $\square$, pH 8.5 enzyme; $\triangle$, pH 12 enzyme.

Fig. 2. Activities of the three phosphatases in regenerating nucleate cells. Caps were removed from cells and enzyme activities measured during regeneration. For each set of assays 50 cells were used. Formation of new caps was complete in 6–8 weeks. $\circ$, pH 5 enzyme; $\square$, pH 8.5 enzyme; $\triangle$, pH 12 enzyme.

is again accompanied by a marked increase in the activity of the pH 12 enzyme.

Synthesis of phosphatases in regenerating enucleate cells. As described in the Materials and Methods section, caps were removed from cells about 35 mm. in length and the cells were then kept in the dark for 8 days before removal of the rhizoids. Regeneration of the cap took place more slowly in the enucleate cells than in those containing nuclei, and a higher proportion (about 60%) of the enucleate cells failed to form a new cap. The cells were studied for a period of 90 days after enucleation, 30–50 cells being taken at suitable intervals for each set of assays. During the first 40 days it was not possible to distinguish between those cells that were going to form a new cap and those that were not, so that during this period samples were taken from the whole population. After 40 days regenerating cells could be distinguished by the appearance of swelling in the growing tips preparatory to the formation of whorls and caps. The regenerating cells were therefore separated from those which were not regenerating and assays were carried out on both groups. The results of the experiment are shown in Fig. 3. It will be seen that there is an increase in acid-phosphatase activity during the course of the experiment, but again the most striking change is the marked increase in pH 12 enzyme activity associated with the re-formation of the cap. This increase did not occur in those cells which failed to regenerate a new cap.

Fig. 3. Activities of the three phosphatases in regenerating and non-regenerating enucleate cells. Regenerating cells: ○, pH 5 enzyme; □, pH 8.5 enzyme; △, pH 12 enzyme. Non-regenerating cells: ●, pH 5 enzyme; ■, pH 8.5 enzyme; ▲, pH 12 enzyme. Caps were removed and the cells enucleated 8 days later. Enzyme activities were measured at 10-day intervals. At 40 days, when regenerating cells could be distinguished, they were separated from the rest, and assays were then done on both types of cells.

DISCUSSION

The present findings demonstrate that in enucleate *Acetabularia* cells increases may occur in the activities of specific enzymes; in one enzyme an increase in activity has been shown to be associated with a particular event in morphogenesis. Two questions arise: (1) does the increase in enzyme activity in the regenerating enucleate cells represent new synthesis of enzyme protein?; (2) are the results obtained in *Acetabularia* applicable to other cells?

An affirmative answer can be given to the first question with some confidence. In the present experiments the activity of all three phosphatases is reduced immediately after removal of the cap. This is due simply to removal of the substantial amounts of these enzymes which are normally present in the cap. When the cap is re-formed the activities of the enzymes return to normal. Since the cap may be removed and regenerated repeatedly over a period of years (Hämmerling, 1963), it is difficult to imagine any process, other than synthesis of the enzymes involved, which could account for the observed results. Increases in the activities of other enzymes have also been reported in enucleate *Acetabularia*. For example, Clauss (1959) has described an increase in phosphorylase activity and Baltus (1959) an increase in aldolase activity. Keek & Clauss (1958) found an increase in invertase activity which continued until the time of cap formation and was then followed by a gradual decrease; they also found that an initial increase in acid-phosphatase activity was followed by a slight decrease. Hämmerling *et al.* (1959) have demonstrated that regeneration in enucleate *Acetabularia* is associated with a progressive net synthesis of protein. There is therefore every reason to believe that the increases in the phosphatase activities described in the present paper do represent new synthesis of these enzymes.

A feature of greater interest is the observation that in both nucleate and enucleate cells synthesis of the pH 12 enzyme is delayed until the cap is about to be formed and then proceeds at a faster rate than the synthesis of the other two phosphatases. This finding clearly indicates that the cytoplasm of *Acetabularia* contains independent mechanisms not only for the synthesis of specific enzymes, but also for the continuous regulation of this synthesis over a period of many weeks.

It is difficult to reconcile these conclusions with the theory that the regulation of cytoplasmic enzyme synthesis is mediated by the flow of metabolically unstable compounds from the nucleus to the cytoplasm; it is therefore of some importance to consider whether *Acetabularia* is exceptional in this respect. A complete answer to this question requires

that comparable experiments be done on other cells, but some experiments which have already been done do, indeed, suggest that the findings in *Acetabularia* may well prove to be widely applicable. For example, Reich, Franklin, Shatkin & Tatum (1962) have shown that, in certain tissue-culture cells, synthesis of protein as measured by the incorporation of radioactive amino acids can proceed at a normal rate for many hours after the synthesis of RNA has been almost totally abolished by the antibiotic actinomycin D. Greengard, Smith & Acs (1963) have shown that the administration of tryptophan will induce the synthesis of tryptophan pyrrolase in liver cells under conditions in which synthesis of RNA in these cells has been largely inhibited by actinomycin D. Rosas del Valle & Aronson (1962) have shown that the synthesis of the proteins necessary for spore formation in *Bacillus cereus* can take place in the normal way at a time when RNA synthesis is suppressed by actinomycin D. These experiments do not, of course, demonstrate that the cytoplasm of these cells possesses the ability to regulate the synthesis of specific proteins independently of the nucleus, as in *Acetabularia*; but all three cases appear to indicate that the regulation of this synthesis does not depend upon the flow of metabolically unstable RNA molecules from the nucleus. Finally, mention must be made of the experiments of McFall & Mandelstam (1963) in *E. coli*. These authors found that the synthesis of β -galactosidase could be repressed by glucose and many of its metabolites even in the so-called 'constitutive' strains of this organism which have mutations at the genetic locus responsible for 'inducibility' of this enzyme. These findings indicate that even in *E. coli* some general mechanism exists for the regulation of enzyme synthesis, which does not involve the immediate activity of the specific genetic locus concerned with this regulation. It may therefore be supposed that many types of cell possess independent cytoplasmic mechanisms for the regulation of enzyme synthesis; and *Acetabularia* may represent, not an exception to the rule, but a striking example of a quite general phenomenon.

SUMMARY

1. *Acetabularia crenulata* contains three phosphatases having pH optima 5.0, 8.5 and 12.0.
2. The synthesis of these enzymes was studied during normal growth of the cells and during the

period of regeneration which follows removal of the cap. A comparison was made between regenerating cells which contained a nucleus and similar cells from which the nucleus had been removed.

3. Synthesis of all three enzymes took place in the enucleate cell.

4. Synthesis of the pH 12 enzyme was found to be specifically associated with the formation of the cap. When the cap was re-formed after amputation, this enzyme was synthesized in similar amounts in the nucleate and the enucleate cell.

5. These findings indicate not only that synthesis of specific enzymes can take place in the absence of the nucleus, but also that this synthesis can be regulated in the enucleate cell.

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REFERENCES

- Baltus, E. (1959). *Biochim. biophys. Acta*, **33**, 337.
 Beth, K. (1953). *Z. Naturf.* **8b**, 334.
 Boman, H. G. (1955). *Biochim. biophys. Acta*, **16**, 245.
 Brenner, S., Jacob, F. & Meselson, M. (1961). *Nature, Lond.*, **190**, 576.
 Clauss, H. (1959). *Planta*, **52**, 534.
 Garen, A. & Levinthal, C. (1960). *Biochim. biophys. Acta*, **38**, 470.
 Goldstein, L., Micou, J. & Crocker, T. T. (1960). *Biochim. biophys. Acta*, **45**, 82.
 Greengard, O., Smith, M. A. & Acs, G. (1963). *J. biol. Chem.* **238**, 1548.
 Gros, F., Hiatt, H., Gilbert, W., Kurland, C. G., Risenbrough, R. W. & Watson, J. D. (1961). *Nature, Lond.*, **190**, 581.
 Hämmerling, J. (1943). *Z. indukt. Abstamm.- u. Vererb.-Lehre*, **81**, 84.
 Hämmerling, J. (1944). *Arch. Protistenk.* **97**, 7.
 Hämmerling, J. (1953). *Int. Rev. Cytol.* **2**, 475.
 Hämmerling, J. (1963). *Annu. Rev. Plant Physiol.* **14**, 65.
 Hämmerling, J., Clauss, H., Keck, K., Richter, G. & Werz, G. (1959). *Exp. Cell Res. suppl.* **6**, 210.
 Hiatt, H. H., Gros, F. & Jacob, F. (1963). *Biochim. biophys. Acta*, **72**, 15.
 Keck, K. & Clauss, H. (1958). *Bot. Gaz.* **120**, 43.
 McFall, E. & Mandelstam, J. (1963). *Nature, Lond.*, **197**, 880.
 Monod, J. & Jacob, F. (1961). *J. molec. Biol.* **3**, 318.
 Prescott, D. M. (1959). *J. biophys. biochem. Cytol.* **6**, 203.
 Prescott, D. M. (1960). *Exp. Cell Res.* **19**, 29.
 Reich, E., Franklin, R. M., Shatkin, A. J. & Tatum, E. L. (1962). *Proc. nat. Acad. Sci., Wash.*, **48**, 1238.
 Rosas del Valle, M. & Aronson, A. I. (1962). *Biochem. biophys. Res. Commun.* **9**, 421.