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Sites of Synthesis of Encephalomyocarditis Virus Components in Infected L-Cells

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SUMMARY

L-cells were fractionated at various times after infection with EMC virus and assayed for haemagglutinin, virus and phenol-extractable infective RNA. A second fraction of infective RNA was obtained by sodium dodecyl sulphate (SDS) extraction of the samples after phenol extraction. The infectivity of SDS-extracted RNA increased earlier than that of phenol-extracted RNA, but the maximum titre was less than 10% of the maximum titre extracted by phenol. The early rise in SDS-extracted RNA was largely confined to the nuclear fraction, and the infectivity of phenol-extracted RNA appeared to increase in the nuclear fraction before the cytoplasmic fraction. There was a simultaneous increase in the titres of both fractions of RNA, haemagglutinin and virus in the cytoplasmic fraction from 3 to 5 hr after infection, and the maximum titres of infective RNA were found in the cytoplasm 4–5½ hr after infection. It was concluded that synthesis of viral RNA begins near the nuclear membrane and then spreads outwards through the cytoplasm, but that there is no conclusive evidence as to whether this process begins in the nuclei or the perinuclear cytoplasm.

INTRODUCTION

Synthesis of viral ribonucleic acid (RNA) 1–2 hr before the appearance of intracellular virus has been reported during replication of mouse encephalomyocarditis (EMC) virus in Krebs-2 ascites tumour cells, and of mouse encephalitis virus in L-cells (Sanders, 1960; Martin & Work, 1962; Hausen & Schäfer, 1961). It was recently suggested that infective RNA was first produced in the nuclei of ascites cells during the 4½ hr after infection with EMC virus, and that the RNA was then transferred to the cytoplasm and there initiated the production of haemagglutinin and plaque-forming virus (Bellett & Burness, 1960, 1963).

In L-cells infected with mengo virus, both acridine orange staining and autoradiography with tritiated uridine indicated that new RNA accumulated in the perinuclear cytoplasm just before the appearance of viral antigen; this suggested that viral RNA was synthesized in the cytoplasm shortly before the viral protein (Franklin, 1962; Franklin & Rosner, 1962; Franklin & Baltimore, 1962). This apparent difference between the replication of EMC virus and mengo virus was unexpected, since the two viruses are very closely related (Dick, 1949; Warren, Smadel & Russ, 1949).

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Dulbecco (1962) thought that such a fundamental difference was unlikely to be real, and noted a similar discrepancy in the apparent sites of synthesis of myxovirus soluble antigens. He proposed that the nucleic acid of all RNA viruses is synthesized in the nuclei of infected cells, any apparent exceptions being due to very rapid transfer of the RNA to the cytoplasm. He concluded that RNA could be transferred from nucleus to cytoplasm fast enough to give Franklin's results, since intercellular transfer of DNA occurs at a similar rate during bacterial conjugation. According to this hypothesis, RNA did not accumulate in the nuclei of L-cells infected by mengo virus because transfer of viral RNA to the cytoplasm was faster in L-cells than in Krebs-2 ascites cells infected by EMC virus, synthesis of RNA being nuclear in both systems.

Experiments reported in this paper were designed to test this hypothesis by a study of the replication of EMC virus in L-cells. The results were consistent with the hypothesis but were not conclusive. Since the work was completed, further evidence for the cytoplasmic synthesis of RNA by small riboviruses has been published (Holland & Bassett, 1964; Horton *et al.* 1964). The present position is briefly reviewed, and it is concluded that RNA synthesis by small riboviruses begins near the nuclear membrane and then spreads outwards through the cytoplasm, but that there is no conclusive evidence as to whether this process begins within the nucleus or in the perinuclear cytoplasm.

METHODS

Growth of virus in L-cells

L-cells (10^8/ml. in Eagle's medium) were infected with 5 plaque-forming units (p.f.u.)/cell of EMC virus (K-2 strain) for 30 min. at 4°, centrifuged for 5 min. at 900 rev./min. and resuspended to 10^7 cells/ml. in warm (37°) Eagle's medium containing 5% (v/v) heat-treated (60°, 30 min.) calf serum. The infected cells were maintained in suspension at 37° and sampled at intervals. Samples were pipetted into about 10 vol. of ice-cold phosphate-buffered saline without calcium or magnesium, centrifuged, washed and either stored at −20° or used for preparation of sub-cellular fractions.

Preparation of subcellular fractions

The fractions were prepared at 0–4°. Infected cells (3 to 5×10^7) were washed with 1 ml. 0·001 M-disodium ethylenediaminetetra-acetate (EDTA, pH 7·0), and then disrupted and separated into nuclear and cytoplasmic fractions by the method of Fisher & Harris (1962). The preliminary wash in hypotonic EDTA decreased to 20 min. the time necessary to obtain a clean nuclear fraction without significantly affecting the recovery of nuclei, although it may have caused some loss of nuclear soluble RNA (Srinivasan, Miller-Faures & Errera, 1962). Small tags of cytoplasm were seen by phase-contrast microscopy on 5–10% of the nuclei. The fractions were stored at −20°.

Preparation of phenol-extracted RNA

RNA was prepared by two phenol extractions in 0·1 M-acetate buffer, pH 6·0, containing 0·05 M-EDTA and 2% bentonite (Fraenkel-Conrat, Singer & Tsugita, 1961). The RNA was twice precipitated by ethanol and stored at 4° in 67% ethanol. RNA samples were dissolved in 1 ml. 0·2 M-phosphate buffer, pH 6·8, just before use.

Sodium dodecyl sulphate (SDS)-extracted RNA

A second fraction of infective RNA can be obtained by SDS extraction of the layer remaining at the interface between the aqueous and phenol layers after the first phenol extraction (Montagnier & Sanders, 1963). The interfaces and the underlying phenol layers were first washed with 5 ml. 0·1 M-acetate buffer, pH 6·0, containing 0·05 M-EDTA, and then shaken with 5 ml. of the same solution containing 2% (w/v) SDS. The preparation was completed by a second phenol extraction of the SDS supernatant fluid, followed by ethanol precipitation of the RNA as before.

SDS-extracted nucleic acid contained deoxyribonucleic acid and RNA, including a fraction rapidly labelled by ^{32}P (Montagnier & Sanders, 1963); 89% of the nucleic acid was derived from nuclei. The bulk of the infective RNA obtained by SDS extraction of infected cells soon after infection had similar properties to the single-stranded viral RNA (Burness *et al.* 1963), but at later times up to 65% of the infectivity was in a form which had properties consistent with a double-stranded structure (Montagnier & Sanders, 1963).

Assay of infectivity

The infectivity of RNA preparations was determined in Krebs-2 ascites cells by the plaque method of Montagnier & Sanders (1962). SDS-extracted RNA showed inhibition of plaque formation at high concentrations; the infective titres were therefore calculated from the highest dilutions giving a mean of 5 or more plaques/plate. Since the variation in recovery of RNA (as measured by extinction at 260 mμ) was small compared with the error in plaque titration of the RNA preparations, the results were expressed simply as p.f.u./ml. of RNA.

Haemagglutinin and virus were assayed by methods previously described (Sanders, Huppert & Hoskins, 1958; Bellett, 1960).

RESULTS

Replication of EMC virus in L-cells

The amount of virus which could be recovered from L-cells fell shortly after infection with EMC virus until, at 2 hr after infection, less than 2% of the cells which registered as infective centres contained detectable plaque-forming virus. New haemagglutinin and virus were first detected at 3 hr after infection, and then increased exponentially until about 6 hr after infection (Fig. 1). Release of virus into the medium began between 4 and 5 hr after infection, and was not complete when the experiments were ended at 7 hr.

Early synthesis of infective RNA

The amount of infective RNA recoverable from L-cells also fell soon after infection, but infective RNA increased in titre 1–2 hr before the virus titre increased (Fig. 1). Infective phenol-extracted RNA increased exponentially from 1 to 5 hr after infection. The infectivity of SDS-extracted RNA seemed to increase more rapidly than the infectivity of phenol-extracted RNA until about 3 hr after infection; this was followed by a slower increase until 5 hr in some experiments. Although SDS-extracted RNA was produced before phenol-extracted RNA it did not accumulate to the same extent (Fig. 2), so that the maximum titre of infective SDS-extracted

RNA was less than 10% of the maximum phenol-extracted RNA titre (mean of 4 experiments 5·7%). The highest proportion of infectivity in RNA extracted by SDS was found at 3 hr.

Intracellular location of SDS-extracted RNA

The early rise in infective SDS-extracted RNA was largely confined to the nuclear fraction (Fig. 3). The infectivity of nuclear SDS-extracted RNA increased from 1 to 4 hr after infection, whereas that of cytoplasmic SDS-extracted RNA did not increase until about 3 hr. At 3 hr 80–97% of the infectivity of SDS-extracted RNA from cell fractions was associated with the nuclear fraction. There was, however, a late increase in the infectivity of cytoplasmic SDS-extracted RNA.

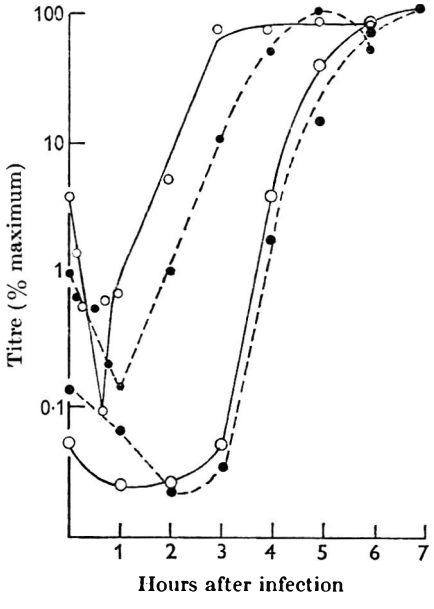

Fig. 1

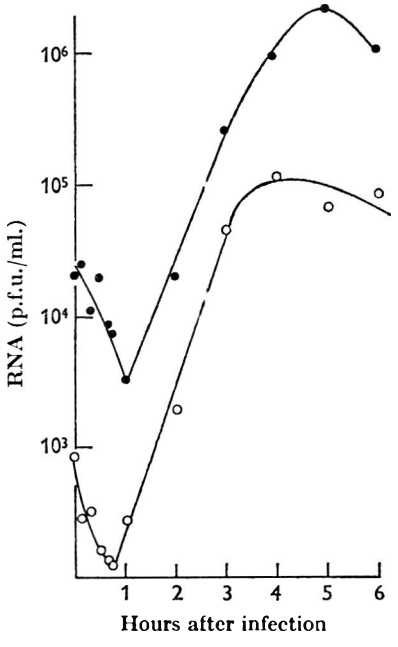

Fig. 2

Fig. 1. Replication of EMC virus components in L-cells. L-cells were infected with EMC virus and incubated in suspension. Samples were removed at intervals and extracted with phenol and then SDS; the RNA preparations were then assayed for infective RNA. Other cell samples were disrupted and assayed for haemagglutinin and plaque-forming virus. The results were expressed as percentages of the maximum yield of each virus component. ○——○, SDS-extracted RNA; ● - - ●, phenol-extracted RNA; ○——○, haemagglutinin; ● - - - ●, plaque-forming virus.

Fig. 2. Infective SDS- and phenol-extracted RNA in infected L-cells. Samples of infected cells were extracted with phenol and then SDS. Each RNA sample was dissolved in 1 ml. 0·2 M-phosphate buffer, pH 6·8, and assayed for infective RNA. ○——○, SDS-extracted RNA; ●——●, phenol-extracted RNA.

Intracellular location of phenol-extracted RNA

There was no long period of accumulation of infective phenol-extracted RNA in the nuclear fraction of L-cells infected by EMC virus, in contrast to our previous results with Krebs-2 ascites cells (Bellett & Burness, 1963); nevertheless, when the results of several experiments were compared it was found that the increase in

infective RNA extracted from the nuclear fraction by phenol occurred consistently 30–60 min. before the increase in cytoplasmic infective RNA (Fig. 4). The infectivity of RNA extracted from the nuclear fraction by phenol exceeded that of cytoplasmic phenol-extracted RNA from 2 to about 4 hr after infection, after which the infective RNA was concentrated in the cytoplasmic fraction. The highest proportion of infectivity in RNA extracted from the nuclear fraction was again found about 3 hr after infection. In two experiments over 90 % of the infectivity of phenol-extracted RNA from cell fractions was found in the nuclear fraction at this time (Fig. 5); in other experiments a smaller proportion of the infectivity was found in the nuclear fraction, the mean value being 78 %. In one experiment only 45 % of the infectivity was nuclear, but the recovery of infective RNA was poor.

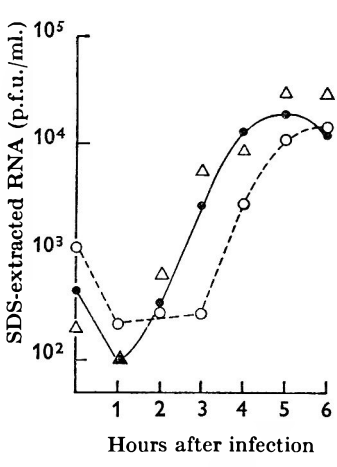

Fig. 3

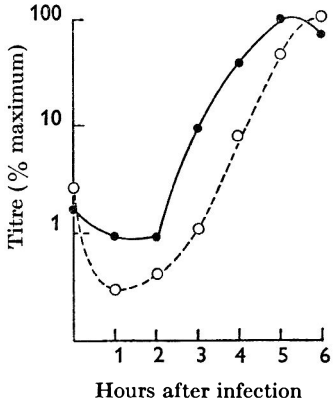

Fig. 4

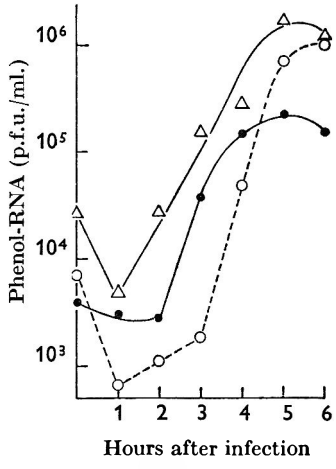

Fig. 5

Figs. 3–5. Location of infective RNA in subcellular fractions of infected L-cells. Samples of infected cells were disrupted and separated into subcellular fractions. The fractions were extracted with phenol and then SDS. Each RNA preparation was dissolved in 1 ml. 0·2 M-phosphate buffer, pH 6·8, and assayed for infective RNA.

Fig. 3. Infective SDS-RNA in subcellular fractions. ●——●, Nuclear SDS-RNA; ○ - - - ○, cytoplasmic SDS-RNA; △, whole cell SDS-RNA.

Fig. 4. Infective phenol RNA in subcellular fractions. Results were expressed as percentages of the maximum titre in each fraction; each point is the mean of four determinations. ●——●, Nuclear phenol-RNA; ○ - - ○, cytoplasmic phenol-RNA.

Fig. 5. Infective phenol RNA in subcellular fractions. The results of a typical experiment expressed as p.f.u./ml. RNA. ●——●, Nuclear phenol-RNA; ○ - - - - ○, cytoplasmic phenol-RNA; △——△, whole cell phenol-RNA.

Recovery of infective phenol-extracted RNA from subcellular fractions was low compared with the amount obtained from whole cells. While it may be concluded that both nuclear and cytoplasmic infective phenol-extracted RNA increased from about 1 to 5 hr after infection, the conclusion that the nuclear increase preceded the cytoplasmic increase depends upon the assumption that the amount of RNA lost from the cytoplasmic fraction did not greatly exceed the amount lost from the nuclear fraction.

Cytoplasmic production of haemagglutinin and plaque-forming virus

Both haemagglutinin and plaque-forming virus were produced in the cytoplasmic fraction from 3 to 6 hr after infection (Figs. 6, 7). Haemagglutinin and virus found in the nuclear fraction during this period was probably due to cytoplasmic contamination of the nuclear fraction, since less than 6% of the haemagglutinin (mean 3·6%) and less than 20% of the virus (mean 14·8%) was nuclear at any one time. A higher proportion of both haemagglutinin and virus was apparently

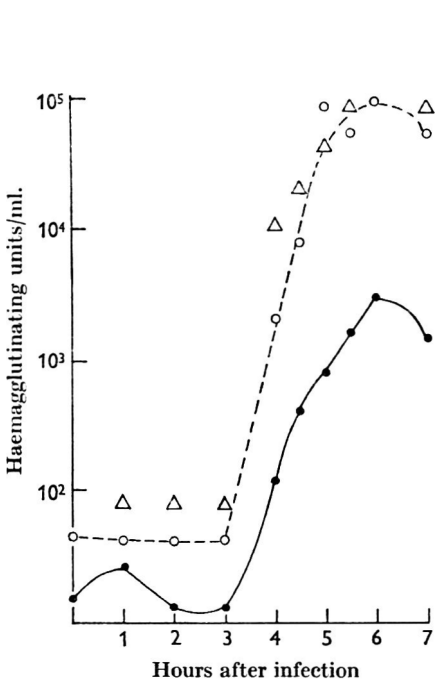

Fig. 6

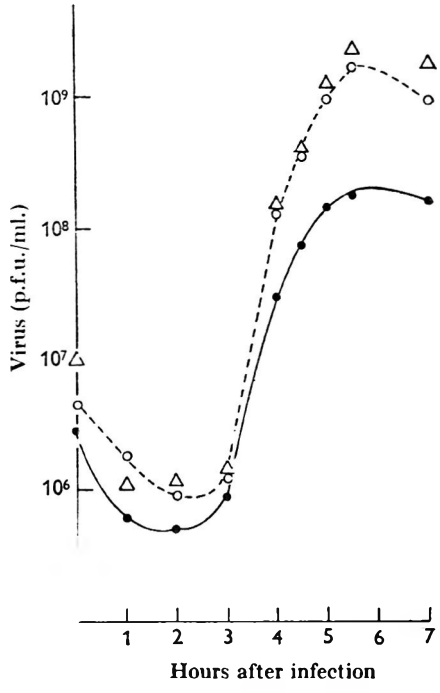

Fig. 7

Figs. 6, 7. Location of haemagglutinnin and virus in subcellular fractions of infected L-cells. Samples of infected cells were disrupted and separated into subcellular fractions. The fractions were further disrupted by shaking with glass beads, clarified and assayed for haemagglutinin and plaque-forming virus.

Fig. 6. Haemagglutinin in subcellular fractions. ●—●, Nuclear haemagglutinin; ○ - - - ○, cytoplasmic haemagglutinin; △, whole cell haemagglutinin.

Fig. 7. Virus in subcellular fractions. ●—●, Nuclear virus; ○ - - - ○, cytoplasmic virus; △, whole cell virus.

nuclear at 3 hr, probably because production of virus began close to the nuclear membrane, so that cytoplasm contaminating the nuclear fraction was particularly rich in virus at this time (Franklin, 1962), but at no time did the proportion of virus in the nuclear fraction approach the high proportion of infective RNA found in that fraction between 2 and 4 hr after infection.

DISCUSSION

Results presented in this paper appear to demonstrate that infective RNA increases in titre in the nuclear fraction of homogenates of L-cells infected by EMC virus earlier than in the cytoplasmic fraction, but that the highest titres of infective RNA are found in the cytoplasmic fraction between 4 and $5\frac{1}{2}$ hr after infection. The rise in infectivity of phenol-extracted RNA is preceded by a rise in SDS-extracted RNA, which is found mainly in the nuclear fraction from 2 to 4 hr after infection. There is an increase in the titre of SDS-extracted RNA in the cytoplasmic fraction from 3 to 5 hr after infection. Most of the total infectivity of RNA is in the phenol-extractable fraction throughout the growth cycle. The increases in cytoplasmic infective RNA concide with the appearance of virus in the cytoplasmic fraction; but the earlier increases in infectivity of RNA extracted from the nuclear fraction do not appear to be associated with haemagglutinin or virus.

We did not purify our nuclear fraction so that we were able to estimate the recovery of infective RNA in subcellular fractions compared with whole cells. This raises the question of whether the infective RNA found in our nuclear fraction was due to cytoplasmic contamination. While we cannot exclude this possibility, to explain our results by cytoplasmic contamination we should have to assume that the perinuclear cytoplasm was much richer in viral RNA than the rest of the cytoplasm during the 3 hr following infection, and that this position was reversed at later times.

Holland & Bassett (1964) showed that contamination by cell membranes probably accounted for the infective RNA found in the nuclear fraction of poliovirus-infected cells under conditions which prevented the virus from replicating. Their evidence for cytoplasmic synthesis of poliovirus RNA is not conclusive, however, since their titres of infective RNA were low, they did not include whole-cell controls to estimate the loss of RNA during fractionation, and they did not demonstrate an increase in titre of cytoplasmic infective RNA during replication of the virus.

Zalta, Rozencwajg, Breugnon & Huppert (1963) observed apparent synthesis of infective RNA by isolated nuclei of cells infected with EMC virus, although Crocker, Pfendt & Spendlove (1964) showed some incorporation of tritiated uridine into poliovirus-infected enucleate fragments of cells after 7 hr exposure to 5 μc/ml. of the label. Experiments in this laboratory (Burness & Bellett, unpublished) did not show unequivocally synthesis of EMC viral RNA by isolated nuclear or cytoplasmic fractions of infected cells.

Several groups have found increases in RNA-dependent RNA polymerase activity after infection of cells with small riboviruses. In some cases this was found in cytoplasmic fractions but no results were reported for the discarded nuclear fractions (Baltimore & Franklin, 1962; Baltimore, Eggers, Franklin & Tamm, 1963). Cline, Eason & Smellie (1963) showed an increase in RNA-dependent polymerase activity in both nuclear and cytoplasmic fractions of cells after infection by EMC virus. Polymerase activity was cytoplasmic in cells fractionated 4–$4\frac{1}{2}$ hr after infection by poliovirus (Holland & Bassett, 1964), and $5\frac{1}{2}$ hr after infection by EMC virus (Horton *et al.* 1964), but results of fractionation experiments at earlier times were not reported.

Levy (1961) showed increased incorporation of uridine into the nucleoli of cells

1–3½ hr after infection by poliovirus, and at about the same time the nuclei of the cells stained brightly with fluorescent antibody. At 4–5 hr after infection, the fluorescence was cytoplasmic. Dunnebacke (1962) found enteroviruses only in nucleate fragments of human amnion cells bisected before 4½ hr after infection, but in other cells both nucleate and enucleate fragments yielded virus. These experiments indicate nuclear involvement in the replication of small riboviruses, but provide no direct evidence that viral RNA is synthesized in nuclei.

Most of the evidence available at present is consistent with the hypothesis that synthesis of the RNA of the small riboviruses begins close to the nuclear membrane, and then spreads outwards through the cytoplasm. There is as yet no conclusive evidence as to whether RNA synthesis begins within the nuclei of infected cells or in the perinuclear cytoplasm.

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REFERENCES

BALTIMORE, D., EGGERS, H. J., FRANKLIN, R. M. & TAMM, I. (1963). Poliovirus induced RNA polymerase and the effects of virus-specific inhibitors on its production. *Proc. natn. Acad. Sci., U.S.A.* **49**, 843.

BALTIMORE, D. & FRANKLIN, R. M. (1962). Preliminary data on a virus-specific enzyme system responsible for the synthesis of viral RNA. *Biochem. biophys. Res. Commun.* **9**, 388.

BELLETT, A. J. D. (1960). Use of Pardee's CO_2 buffer in plaque titration of EMC virus. *Virology*, **10**, 285.

BELLETT, A. J. D. & BURNESS, A. T. H. (1960). Sites of synthesis of a nucleic acid and a protein in infected Krebs-2 ascites tumour cells. *Biochem. J.* **77**, 17P.

BELLETT, A. J. D. & BURNESS, A. T. H. (1963). Intracellular sites of synthesis of encephalomyocarditis virus components in Krebs-2 ascites tumour cells. *J. gen. Microbiol.* **30**, 131.

BURNESS, A. T. H., VIZOSO, A. D., CLOTHIER, F. W., MONTAGNIER, L. & SANDERS, F. K. (1963). Encephalomyocarditis virus and its ribonucleic acid: sedimentation characteristics. *Nature, Lond.* **197**, 1177.

CROCKER, T. T., PFENDT, E. & SPENDLOVE, R. (1964). Poliovirus: growth in non-nucleate cytoplasm. *Science*, **145**, 401.

CLINE, M. J., EASON, R. & SMELLIE, R. M. (1963). The biosynthesis of ribonucleic acid following infection with a ribonucleic acid virus. *J. biol. Chem.* **238**, 1788.

DICK, G. W. A. (1949). The relationship of mengo encephalomyelitis, encephalomyocarditis, Columbia-SK and M.M. viruses. *J. Immunol.* **62**, 375.

DULBECCO, R. (1962). Basic mechanisms in the biology of animal viruses. *Cold Spr. Harb. Symp. quant. Biol.* **27**, 519.

DUNNEBACKE, T. H. (1962). Amounts of polio and coxsackie viruses within the separate portions of bisected cultured cells. *Virology*, **16**, 392.

FISHER, H. W. & HARRIS, H. (1962). The isolation of nuclei from animal cells in culture. *Proc. Roy. Soc.* B, **156**, 521.

FRAENKEL-CONRAT, H., SINGER, B. & TSUGITA, A. (1961). Purification of viral RNA by means of bentonite. *Virology*, **14**, 54.

FRANKLIN, R. M. (1962). A cytochemical description of the multiplication of mengo virus in L-929 cells. *J. Cell Biol.* **12**, 1.

FRANKLIN, R. M. & BALTIMORE, D. (1962). Patterns of macromolecular synthesis in normal and virus-infected mammalian cells. *Cold Spr. Harb. Symp. quant. Biol.* **27**, 175.

FRANKLIN, R. M. & ROSNER, J. (1962). Localization of ribonucleic acid synthesis in mengo virus-infected cells. *Biochim. biophys. Acta*, **55**, 240.

HAUSEN, P. & SCHÄFER, W. (1961). Production of virus RNA and virus antigen during multiplication of the ME virus. *Z. Naturf.* **166** b, 72.

HOLLAND, J. J. & BASSETT, D. W. (1964). Evidence for cytoplasmic replication of poliovirus ribonucleic acid. *Virology*, **23**, 164.

HORTON, E., LIU, S. L., DALGARNO, L., MARTIN, E. M. & WORK, T. S. (1964). Development of ribonucleic acid polymerase in cells infected with encephalomyocarditis virus and the synthesis of single- and double-stranded RNA by the isolated polymerase. *Nature, Lond.* **204**, 247.

LEVY, H. B. (1961). Intracellular sites of poliovirus reproduction. *Virology*, **15**, 173.

MARTIN, E. M. & WORK, T. S. (1962). Studies on protein and nucleic acid metabolism in virus-infected mammalian cells. 5. The kinetics of synthesis of virus protein and virus ribonucleic acid in Krebs II mouse ascites-tumour cells infected with encephalomyocarditis virus. *Biochem. J.* **83**, 574.

MONTAGNIER, L. & SANDERS, F. K. (1962). Titrage de l'acide ribonucléique infectieux du virus de l'encéphalomyocardite de la souris sur cellules d'ascite Krebs II *in vitro*. *C.r. hebd. Séanc. Acad. Sci., Paris*, **254**, 2247.

MONTAGNIER. L. & SANDERS, F. K. (1963). The replicative form of encephalomyocarditis virus ribonucleic acid. *Nature, Lond.* **199**, 664.

SANDERS, F. K. (1960). Role of infective nucleic acid in the production of encephalomyocarditis virus. *Nature, Lond.* **185**, 802.

SANDERS, F. K., HUPPERT, J. & HOSKINS, J. M. (1958). Replication of an animal virus. *Symp. Soc. Exp. Biol.* **12**, 123.

SRINIVASAN, P. R., MILLER-FAURES, A. & ERRERA, M. (1962). Isolation techniques for HeLa-cell nuclei. *Biochim. biophys. Acta*, **65**, 501.

WARREN, J., SMADEL, J. E. & RUSS, S. B. (1949). The family relationship of encephalomyocarditis, Columbia-SK, M.M., and mengo encephalomyelitis viruses. *J. Immun.* **62**, 387.

ZALTA, J. P., ROZENCWAJG, R., BREUGNON, M. M. & HUPPERT, J. (1963). Synthèse du RNA infectieux du virus EMC par des noyaux de cellules d'ascite de souris. *C.r. hebd. Séanc. Acad. Sci., Paris*, **256**, 2471.

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Alkaline Phosphatase in *Dictyostelium discoideum*

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SUMMARY

Several phosphatases have been demonstrated in *Dictyostelium discoideum*. The alkaline phosphatase, pH optimum 9·0, is a 5′-nucleotidase, attacking adenosine monophosphate and deoxyadenosine monophosphate, but catalyses also hydrolysis of *p*-nitrophenyl phosphate. In the absence of exogenous nutrients this enzyme increases about 6-fold *in vitro* during differentiation. Its activity *in vivo* may in part be controlled through end-product inhibition by orthophosphate which has been found to accumulate in the cells during sporulation. Exogenous orthophosphate and glucose repress the levels of alkaline phosphatase in the spores. The data support the conclusion that end product inhibition and repression collaborate to ensure maximal alkaline phosphatase activity *in vivo* during culmination. Exogenous adenosine and deoxyadenosine increase the alkaline phosphatase levels in the sorocarps.

INTRODUCTION

Histochemical and *in vitro* studies on the differentiating cellular slime mould *Dictyostelium discoideum* have shown that the enzyme alkaline phosphatase changes in activity during the development of the organism. The activity increases until spore formation but decreases to a minimum in the mature fruiting bodies or sorocarps (Bonner, Chiquoine & Kolderie, 1955; Krivanek, 1956). These studies were performed with β-glycerophosphate as substrate at pH 9·3. With adenylic acid at pH 8·3, Krivanek & Krivanek (1958) found a similar activity pattern for 5′-nucleotidase. It has been shown (Wright, 1960) for other slime mould enzymes that apparent increases in *in vitro* activities during differentiation may be due to rapid inactivation in extracts prepared at the early stages of development and thus do not represent true increases in enzyme concentrations. In the event that true increases are shown to occur, these do not necessarily reflect increased enzyme activities *in vivo*, where, e.g. changes in substrate and inhibitor concentrations may control the reactions. The present investigation was, therefore, undertaken to determine if the observed changes in alkaline phosphatase activity *in vitro* reflect the conditions inside the living cells.

During this work a number of phosphatases have been detected, distinguishable from each other through pH optima, Mg^{2+} requirements and their *in vitro* activities at the various stages of differentiation, They include, besides the alkaline phosphatase, one acid phosphatase, optimum about pH 3·5, one ATP (see p. 312) attacking enzyme, two pyrophosphatases with optima at pH 6·2 and 8·8 and one enzyme,

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active on 5′-GMP, with optimum at pH 7·5–8·5. The present paper will describe the alkaline phosphatase and only include the other enzymes when necessary for distinguishing purposes. The other phosphatases will be dealt with in subsequent papers. For a full description of the life cycle of the slime mould the reader is referred to Bonner's monograph, *The Cellular Slime Molds* (1959). A summary is presented in Fig. 1.

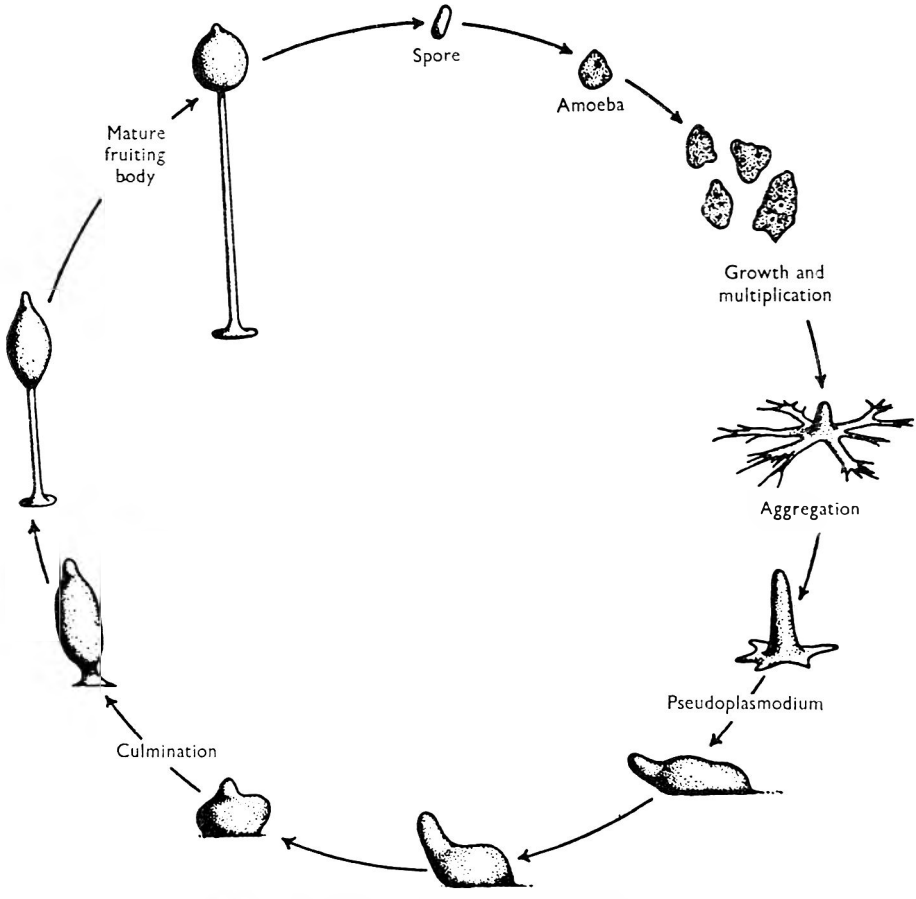

Fig. 1. Life cycle of *Dictyostelium discoideum*.

METHODS

The myxamoebae of *Dictyostelium discoideum* strain NC-4 were grown at 23° on SM agar (Sussman, 1961) with *Escherichia coli* strain ML 304d, washed and transferred to 2% plain agar for starvation and multicellular differentiation at 17° as described by Liddell & Wright (1961). Compounds studied in induction and repression experiments were included in the 2% agar. For comparison, in one experiment the slime mould was allowed to differentiate on the complex medium in the presence of residual food bacteria.

The enzyme activities were studied in crude cell-free extracts. At the desired stage of development the slime mould was harvested from the agar with 0·01 M-tris

buffer (pH 7·2). The suspension was homogenized in an Aminco (American Instrument Corporation, Silver Spring, Md, U.S.A.) French pressure cell at 5000 lb./sq. in. and the homogenate centrifuged at 3000 *g*. for 10 min. All these procedures were undertaken in the cold. The protein content in the extracts was usually about 10 mg./ml.

Protein was determined by the modified micro-biuret method (Zamenhof, 1957) with crystalline serum albumin as a standard. The measurements were performed in a Zeiss spectrophotometer Model PMQ II. The determinations were made either on the crude extracts or after precipitation of the protein in 3·5% (w/v) perchloric acid followed by dissolving of the precipitate in 0·5 M-NaOH. The latter procedure was necessary when the experiments involved a comparison between different developmental stages because of the varying size of the amino acid pool and of the small peptide pool during differentiation (Wright, 1964).

Intracellular orthophosphate. To avoid washing out phosphate from the cells during the harvesting procedure, at different stages the organism was removed from the agar surface with a glass slide and suspended in cold 0·01 M-tris (pH 7·2). At the sorocarp stage determinations were made both on the whole fruiting bodies and on the spores.

A sample of the fresh suspension was immediately extracted for 30 min. in cold 5% trichloroacetic acid (TCA). After centrifugation the orthophosphate in the supernatant fluid was determined according to the method of Fiske & SubbaRow (1925) as modified by Dryer, Tammes & Routh (1957).

Another sample of the suspension was centrifuged for 10 min. at 1000 *g*, giving the corresponding volume of packed organism (Wright & Bard, 1963). The intracellular phosphate concentration was expressed as molarity in terms of packed cell volume.

Enzyme assays. The alkaline phosphatase activity was assayed in diluted extracts (about 0·1 mg. protein/ml.) with *p*-nitrophenyl phosphate (NPP) as substrate (Torriani, 1960) unless specified otherwise. The reaction was followed spectrophotometrically (Beckman DU or Zeiss PMQ II) at 23° by the formation of nitrophenol (NP) at 420 mμ.

The reaction mixture, 1 ml., was 0·05 or 0·5 M-tris (pH 9), 0·02 M-$MgCl_2$, 0·01 M-NPP. In the early experiments, where 0·5 M-tris was used, no $MgCl_2$ was added, and it was also omitted in the experiments on phosphate inhibition. The amount of crude enzyme added corresponded to 5–50 μg. protein/ml. The enzyme-specific activity was calculated from the initial rapid reaction rate and expressed as a μMmole NP/min./mg. protein. Figs. 7–11 express activity in these terms; comparative data on substrate specificity, pH optima, etc., are expressed as per cent of maximal activity.

The hydrolysis of other phosphate compounds was determined as the amount of orthophosphate (Pi) released, and the specific activity expressed as μMmole Pi/min./mg. protein. The reaction mixture was 0·05 M-tris, 0·02 M-$MgCl_2$ (except as noted) and 0·01 M with respect to substrate concentration. The incubation time was 15 min. to 1 hr, occasionally longer, and the temperature was 23°. The reaction was stopped through addition of cold 30% TCA to final concentration 6%. The Pi was determined according to Dryer *et al.* (1957). Control tubes without enzyme were incubated in all experiments.

The hydrolysis of 5′-AMP was determined also as the amount of adenosine formed (Herman & Wright, 1959). The reaction was stopped by addition of 10% TCA to final concentration 2%. After centrifugation, samples (0·03 ml.) of the supernatant fluid were assayed with adenosine deaminase in a total of 1·1 ml. of 0·05 M-phosphate buffer (pH 7·5). The reaction was followed as the decrease in extinction at 265 mμ in the Zeiss (Kornberg & Pricer, 1951).

Reagents. NPP, NP, βGP, G6P, G1P, FDP, 5′-UMP, 5′-CMP, GTP (type II, 95–98%), GDP, UDP, adenosine, deoxyadenosine, and adenosine deaminase (type II, crude powder) were obtained from Sigma Chemical Company, St Louis, Mo, U.S.A.; 5′-GMP, 5′-TMP, ADP and UTP from Pabst Laboratories, Inc., Milwaukee, Wisc., U.S.A.; 5′-AMP and ATP from Sigma and Pabst; 5′-IMP and 5′-dAMP from California Corporation for Biochemical Research, Los Angeles, Calif., U.S.A.; and 3′-AMP from Schwarz BioResearch, Inc., Mount Vernon, N.Y., U.S.A. With the exception of GTP and adenosine deaminase, all compounds were reagent grade.

The following abbreviations are used throughout this paper: NPP, *p*-nitrophenyl phosphate; NP, nitrophenol; Pi, orthophosphate; βGP, β-glycerophosphate; G6P, glucose-6-phosphate; G1P, glucose-1-phosphate; FDP, fructose-1,6-diphosphate; 5′-AMP, 5′-GMP, 5′-CMP, 5′-UMP, 5′-TMP, and 5′-IMP, the 5′-monophosphates of adenosine, guanosine, cytidine, uridine, thymidine and inosine respectively; 5′-dAMP, deoxyadenosine-5′-monophosphate; 3′-AMP, adenosine-3′-monophosphate; ATP, GTP, UTP, ADP, GDP, UDP, the tri- and diphosphates of adenosine, guanosine and uridine; PPi, pyrophosphate; tris, tris(hydroxymethyl)aminomethane; and TCA, trichloroacetic acid.

RESULTS

The stages usually studied were amoeba (starved for 2–3 hr), pre-culmination, culmination and sorocarp (about 10–15 hr after sorocarp formation). The time required for sporulation varied between the experiments depending on the status of the amoebae at the time of transfer from the rich medium, variations in washing and plating procedures, and so on.

pH optimum. As shown in Fig. 2 optimal phosphatase activity against NPP was obtained at pH 9·0 in extracts prepared either at the amoeba or sorocarp stage. The optimum on 5′-AMP was also pH 9, whereas activity on 5′-GMP had a broader optimum around pH 8. This fact and other data (see below) indicate that 5′-GMP is not a substrate for alkaline phosphatase.

Substrate concentration. The effect of increasing substrate concentration on phosphatase activity against NPP and 5′-AMP is shown in Fig. 3. The optimal concentration range was the same for both compounds.

Mg^{2+} activation. The phosphatase was more dependent on Mg^{2+} ions when assayed in a final concentration of 0·05 M-tris than when 0·5 M-tris was used. With dialysed extracts about 75% of maximal activity against NPP was obtained in 0·5 M-tris without addition of Mg^{2+} compared to 50% at the lower buffer concentration (Fig. 4). A similar relationship between buffer concentration and Mg^{2+} activation has been reported for the alkaline phosphatase of *Escherichia coli* (Garen & Levinthal, 1960). Optimal Mg^{2+} concentration in 0·05 M-tris was 0·02–0·05 M at

amoeba and sorocarp stages, with either NPP or 5′-AMP as substrate. (In the curve for AMP the activity without Mg^{2+} is higher than that usually obtained as in Table 3.) In contrast, activity on 5′-GMP was completely dependent on Mg^{2+} implicating another enzyme (see below).

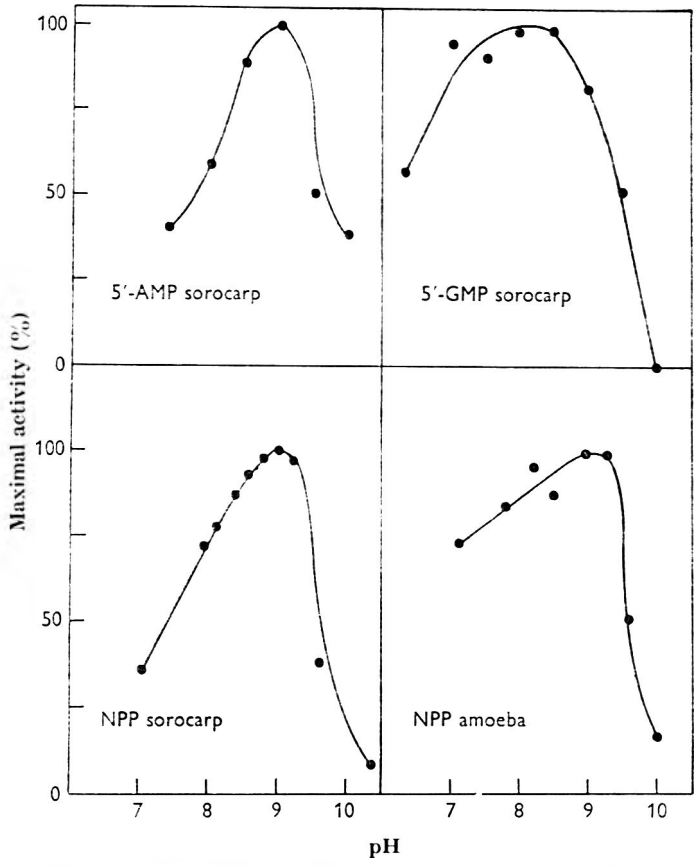

Fig. 2. Effect of pH on phosphatase activity against NPP (bottom), 5′-AMP and 5′-GMP (top). The activities against NPP were assayed in 0·5 M-tris without $MgCl_2$, those against 5′-AMP and 5′-GMP in 0·05 M-tris, 0·02 M-$MgCl_2$. They are expressed in percent of maximal activity obtained.

Distribution of enzyme activity in vitro *during differentiation*. The alkaline phosphatase activity *in vitro* increased about 5–7 times during differentiation. The activity was highest in the sorocarps and the major increase took place during culmination (Table 5).

Since some bacteria were always transferred to the plain agar with the amoebae, and since the amoebae, even after starvation for 2–3 hr, still might contain engulfed *Escherichia coli* in various states of disintegration, the phosphatase activity measured in the amoeba extracts might have been derived in part from bacterial enzyme. No phosphatase activity, however, was demonstrable in the *E. coli* mutant strain used when it was grown on the rich medium. This was consistent with the

finding that alkaline phosphatase formation in *E. coli* strain ML was repressed (Torriani, 1960) by orthophosphate, a component in the SM agar.

In cell-free extracts prepared from the amoebae immediately after removal from the complex medium, as well as after washing and after starvation for 3 hr on the plain agar, the alkaline phosphatase activities were 11, 13 and 19 mμM/min./mg. protein, respectively. In a separate experiment, the amoebae were grown on dead

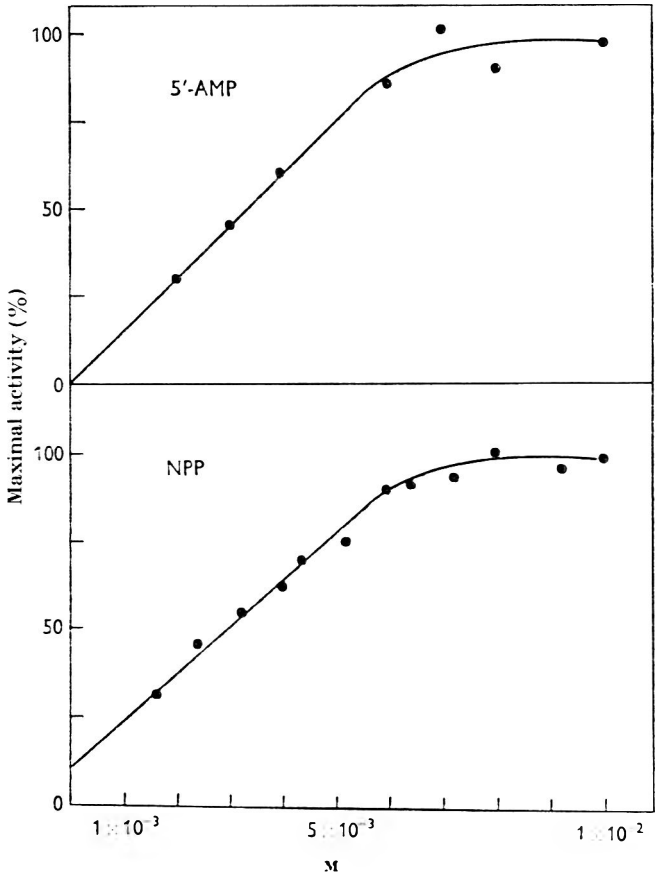

Fig. 3. Phosphatase activity against NPP and 5'-AMP (top) as a function of substrate concentration (same abscissa for each substrate). The activities (expressed as per cent of maximal activity) were assayed in 0·5 M-tris without $MgCl_2$ (NPP) and in 0·05 M-tris, 0·02 M-$MgCl_2$ (AMP).

Escherichia coli. No control with living bacteria was possible because of the lower growth rate on the dead organism (Raper, 1951). The phosphatase activity obtained was 18 mμM/min./mg. protein, i.e. of the same magnitude as observed for amoebae grown on live bacteria. It is therefore concluded that the alkaline phosphatase activity measured in the amoeba extracts was derived from *Dictyostelium discoideum*.

Remnant bacteria, possibly de-repressed on the plain agar, did not contribute significantly to the alkaline phosphatase activity in the sorocarp extracts since spores separated from stalks, debris and bacteria gave an extract higher in specific

activity than the complete sorocarps. Apparently the stalks contained relatively little of the enzyme. All experiments were performed with cell-free extracts. However, the enzyme-specific activity against NPP was the same whether the homogenate or the extract was used.

Stability. Storage of the cell-free extracts at +4° resulted in loss of activity, especially in those prepared at the early stages of development (Table 1). However,

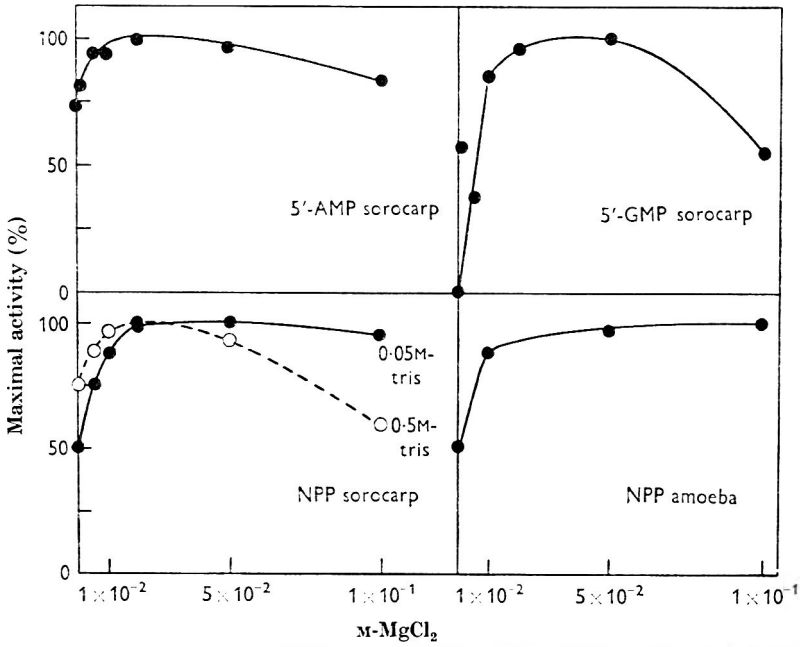

Fig. 4. Mg^{2+} activation of phosphatase activity against NPP (bottom), 5′-AMP and 5′-GMP (top). The tris buffer concentration was 0·05 M. The activity of the sorocarp enzyme against NPP was also assayed in 0·5 M-tris. The activities are expressed as per cent of maximal activity.

Table 1. *Activity with time of undiluted extracts*

	Specific enzyme activities			
Stage	Fresh	1 day	2 days	8 days
Amoeba	10	4	2	1
Preculmination	14	7	3	4
Sorocarp	60	63	37	31

The concentrated extracts were stored at +4° and assayed without dilution in 0·5 M-tris without $MgCl_2$. Specific enzyme activity is defined in Methods.

when the extracts were diluted immediately by a factor of 100, i.e. to a concentration of about 0·1 mg. protein/ml. and stored as such at +4°, no decrease in activity appeared in 48 hr (Table 2). There was, on the contrary, a pronounced increase in alkaline phosphatase activity during the first day, especially in the sorocarp extract (see below). Because of this release of enzyme activity and the stability of the enzyme thereafter, the phosphatase activities were always assayed after storage of

the diluted extracts at +4° for 20–24 hr. Dialysis of the diluted extracts did not increase the alkaline phosphatase activities beyond what was obtained through dilution only.

The crude extracts tolerated a temperature of 50° for 5 min.; prolonged treatment (30 min.) inactivated the alkaline phosphatase. At 80° all activity was lost within 1 min. both at the sorocarp and the amoeba stage. Because of the undefined conditions in the extracts, no further experiments on heat stability were performed.

Table 2. *Effect of dilution and stability of diluted enzyme*

	Specific enzyme activities			
		Diluted extract		
Stage	Undiluted extract	0 hr*	20 hr*	48 hr*
Amoeba	13	16	18	18
Preculmination	28	32	38	37
Sorocarp	52	69	95	95

The fresh extracts were diluted to a concentration of *ca.* 0·1 mg. protein/ml. and stored at +4°. The activities were measured in 0·5 M-tris without $MgCl_2$.

* Hours after initial dilution.

Table 3. *Relative substrate specificities in an extract of sorocarps*

	0·02 M-$MgCl_2$	No $MgCl_2$		0·02 M-$MgCl_2$	No $MgCl_2$
NPP	100	52	5′-UMP	1	—
5′-AMP	41	22	5′-TMP	5	—
5′-GMP	160	0	5′-IMP	1	—
ATP	64	33	3′-AMP	8	—
ADP	5	0	TPN	5	—
GTP	7	0	G6P	5	—
GDP	77	0	G1P	5	—
UTP	45	0	FDP	0	—
UDP	6	0	βGP	5	—
5′-CMP	1	—			

The activities were measured at pH 9·0 in 0·05 M-tris, at 0·01 M substrate concentration.

Substrate specificity

A large number of phosphate esters were tested as substrates for the alkaline phosphatase at the sorocarp and amoeba stages (Tables 3, 4). The tables show that, for the compounds tried at both stages, the substrate specificity was the same in the amoeba and sorocarp extracts. Since the pH optimum (Fig. 2) and the Mg^{2+} activation (Fig. 4) also were the same at both stages, it seemed probable that the amoeba and sorocarp alkaline phosphatases were identical.

βGP, used by Bonner *et al.* (1955) and by Krivanek (1956) was a poor substrate for the enzyme. It required prolonged incubation times (1–3 hr) and higher substrate concentration than did NPP. Although the results for βGP are therefore difficult to interpret, the specific activity did increase from amoeba to sorocarp in the experiments, thus resembling the activity pattern against NPP.

G6P, G1P and FDP were only very slightly hydrolysed at pH 9·0, if at all. Of these compounds, at least G6P and FDP served as substrates for the acid phos-

phatase, which was not activated by Mg^{2+}. The low activities against these three phosphate esters obtained at pH 9·0 were probably due to this latter enzyme.

Of the 5′-nucleotides listed in Table 3, only 5′-AMP and 5′-GMP were readily hydrolysed at pH 9·0; GMP, however, was only attacked in the presence of Mg^{2+}.

With NPP and 5′-AMP there was a 6-fold increase in enzyme concentration during differentiation (Table 5). Hence the ratio between the specific activities in AMP and NPP was constant during development. The specific activity on 5′-GMP was entirely different: it was about the same at all stages. The ratio 5′-GMP:NPP was therefore highest at the amoeba and lowest at the sorocarp stage. Together

Table 4. *Relative substrate specificities in an extract of amoebae*

NPP	100
5′-AMP	42
5′-UMP	0
5′-TMP	0
5′-IMP	0
3′-AMP	0
G6P	0
G1P	0
FDP	9
βGP	14

The activities were assayed at pH 9·0 in 0·05 M-tris, 0·02 M with respect to $MgCl_2$, at 0·01 M substrate concentration.

Table 5. *Phosphatase specific activities on NPP, 5′-AMP and 5′-GMP at four stages of development*

Stage	NPP	5′-AMP	Ratio $\frac{\text{5′-AMP}}{\text{NPP}}$	5′-GMP	Ratio $\frac{\text{5′-GMP}}{\text{NPP}}$
Amoeba	29	11	0·38	187	6·43
Preculmination	56	23	0·41	178	3·17
Culmination	76	29	0·38	157	2·06
Sorocarp	157	66	0·42	176	1·12

The activities were measured at pH 9·0 in 0·05 M-tris, 0·03 M with respect to $MgCl_2$, at 0·01 M substrate concentration.

Table 6. *Phosphate specific activities on NPP, 5′-AMP and 5′-dAMP at two stages of development*

Stage	NPP	5′-AMP	5′-dAMP
Amoeba	24	12	11
Sorocarp	168	79	79

The extracts were assayed in the presence of 0·05 M-tris and 0·02 M-$MgCl_2$, at 0·01 M substrate concentration.

with the absolute Mg^{2+} requirement for phosphatase activity against GMP, the enzyme activity pattern during development provided strong evidence that 5′-GMP was hydrolysed by an enzyme other than the alkaline phosphatase. This conclusion is supported further by the fact that the hydrolysis of GMP has a different pH optimum than that of NPP (Fig. 2).

The phosphatase activity was the same whether 5′-AMP or 5′-dAMP was used

as substrate at pH 9. For both these compounds, as well as for NPP, there was a 7-fold increase in enzyme concentration during differentiation (Table 6). The hydrolysis of 5′-dAMP showed the same Mg^{2+} activation as that of 5′-AMP and NPP. Both AMP and dAMP must thus be considered as probable substrates for the alkaline phosphatase.

Of the di- and trinucleotides studied (Table 3), phosphatase activity was obtained against GDP, ATP and UTP. GDP and UTP were, however, only hydrolysed in the presence of Mg^{2+}. They must therefore be excluded as substrates for the alkaline phosphatase.

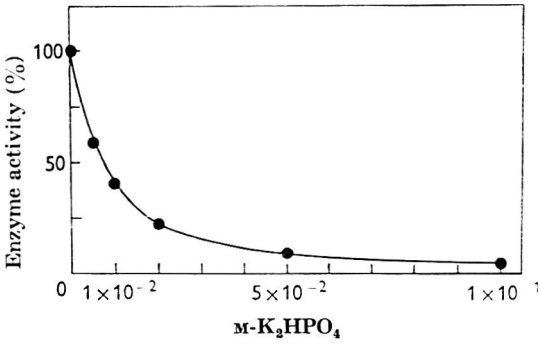

Fig. 5

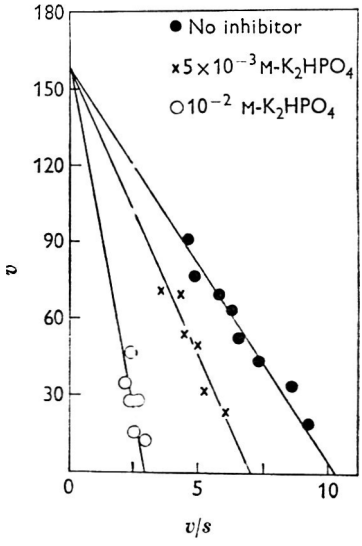

Fig. 6

Fig. 5. Pi inhibition of alkaline phosphatase activity in a dialysed sorocarp extract. The activities were assayed in 0·5 M-tris without $MgCl_2$ and expressed as per cent of the activity obtained in the absence of Pi. Enzyme activity was determined by nitrophenol formation.

Fig. 6. Alkaline phosphatase activity against NPP in a dialysed sorocarp extract assayed with and without added K_2HPO_4. The activities are plotted as *v versus v/s* (*v* in mμM/min./mg. protein; *s* in mM). The activities were measured in 0·5 M-tris without $MgCl_2$.

Table 7. *Phosphatase specific activities of a fresh and aged dialysed extract of sorocarps on NPP, 5′-AMP and ATP*

	Fresh	Aged	Residual activity (%)
NPP	138	131	95
5′-AMP	55	55	100
ATP	37	9	24

The activities were assayed in the presence of 0·05 M-tris, and 0·02 M-$MgCl_2$. The diluted and dialysed extract was aged at −20° for 3 days before re-assaying.

ATP was hydrolysed even in the absence of Mg^{2+} (Table 3). The activity varied immensely, however, between the experiments. As shown in Table 7, the enzyme attacking ATP is very unstable, and it is for this reason not identical with the alkaline phosphatase.

Sodium pyrophosphate, PPi, was hydrolysed at alkaline pH values with a sharp optimum at pH 8·8. The reaction was completely Mg^{2+} dependent and required a Mg:PPi ratio of unity for optimal activity. The specific activity hardly changed during differentiation. (Under optimal conditions the values 1020, 770, 1050 and 970 mμM/min./mg. protein were obtained for the amoeba, pre-culmination, culmination and sorocarp stages, respectively.) Hence the pyrophosphatase and the alkaline phosphatase must be two different enzymes.

Of all naturally occurring compounds studied, only 5′-AMP and 5′-dAMP were possible substrates for the alkaline phosphatase.

With respect to enzyme pattern during differentiation (Table 5) stability (Table 7), pH optimum (Fig. 2), effect of substrate concentration (Fig. 3), and Mg^{2+} activation (Fig. 4) the characteristics for phosphatase activity against NPP and 5′-AMP were the same. This suggests the same enzyme. It is hence concluded that 5′-AMP and 5′-dAMP as well as NPP are hydrolysed by the alkaline phosphatase in *Dictyostelium discoideum*.

Inhibition by orthophosphate (Pi)

With NPP or with 5′-AMP as substrate the alkaline phosphatase in Dictyostelium was inhibited by its end product, orthophosphate. The inhibition at pH 9 was about 50% at a phosphate concentration of 0·007 M and almost complete in 0·05–0·1 M-K_2HPO_4 (Fig. 5). Similar curves were obtained for both amoeba and sorocarp extracts.

In Fig. 6 the enzyme activities with and without phosphate are plotted as *v versus v/s* according to the formula

$$v = V_{max} - v/s \left(1 + \frac{I}{K_I}\right) K_m$$

(Eadie, 1952). The increased slopes in the presence of inhibitor and the fact that the straight lines all have the same intercept (V_{max}) show that orthophosphate was a competitive inhibitor of alkaline phosphatase *in vitro*.

Intracellular orthophosphate

Because of this end-product inhibition of alkaline phosphatase *in vitro*, the intracellular orthophosphate (Pi) concentration was determined at various stages of development. The first column in Table 8 shows that the phosphate concentration increased about 20-fold during the development from amoebae to spores. Calculated for the whole sorocarp the increase was less; the stalks contribute significantly to the packed cell volume but probably not to the phosphate determinations. The main increase in intracellular phosphate occurred at the late culmination and young sorocarp stages. The extent to which the various phosphate concentrations inhibited alkaline phosphatase activity *in vitro* (Fig. 5) is shown in the second column of Table 8.

Phosphate release and phosphate inhibition in vitro

As shown in Table 8 orthophosphate (Pi) was present in the spores in a concentration high enough to inhibit the alkaline phosphatase by more than 90%. The Pi concentration in the amoebae would cause only a 25% inhibition.

In the concentrated fresh extracts, the enzyme must also have been inhibited to

some extent. A determination of the Pi contents gave the values 10^{-3}M for the amoeba and 3×10^{-3}M for the sorocarp extract, corresponding to 10 and 25% inhibition of the enzyme (Fig. 5). A 100-fold dilution of the extracts decreased the Pi values to 10^{-5} and 3×10^{-5}M respectively, at which concentrations there is no Pi inhibition (Fig. 5). However, maximal activity was not obtained immediately after dilution and the increase in the sorocarp extract during the first 20 hr was considerably higher than in the extracts from the early stages (Table 2). In an experiment involving two sorocarp extracts, one was prepared in 0·05 M phosphate

Table 8. *Intracellular Pi molarity* during development*

Stage	Molarity Pi	Inhibition *in vitro* of alkaline phosphatase (%)
Amoeba	0·003	25
Pre-culmination	0·003	25
Culmination	0·006	45
Very young sorocarp (including stalks)†	0·014	67
1 day soroacarp (with stalks)†	0·020	78
Spores	0·065	93

* Expressed in terms of packed cell volume.

† Stalks, present in both these preparations, contribute significantly to the packed cell volumes but not to the phosphate contents of the extracts.

buffer (the approximate Pi concentration in the spores) and the other, as a control, in 0·01 M-tris, both at pH 7·2. The fresh extracts were diluted 100-fold in tris. This brought the Pi concentrations below inhibiting values (Fig. 5) in both extracts. Most of the 90% inhibition caused by the 0·05 M-phosphate was released immediately after dilution. The specific activity was 74 mμm/min./mg. protein compared to 85 in the control. Maximal activity (105 mμM/min./mg. protein) was obtained in both diluted extracts within 20 hr. When the phosphate-containing thick extract was preincubated 24 hr before dilution, the specific activity immediately after dilution was low, 29 mμM/min./mg. protein, suggesting a binding of phosphate to the enzyme. The final activity, after 20 hr, however, was the same as in the not pre-incubated, 100-fold diluted extracts. This slow recovery from Pi inhibition in the preincubated extract may explain the slow increase in activity obtained during the first 20 hr in the diluted ordinary extracts (Table 2). This might then be due to release from bound phosphate (Schwartz, 1963).

Table 1 showed that the phosphatase activity decreased with time in the concentrated extracts. This decrease was about 80% during the first 2 days at the amoeba stage and 40–50% at the sorocarp stage. During this time the phosphate content increased to about 5×10^{-3}M, both in the amoeba and sorocarp extracts. This concentration inhibited the alkaline phosphatase *in vitro* to 40% (Fig. 5), while the Pi concentrations in the fresh extracts corresponded to 10 and 25% inhibition (see above). Hence, only part of the decrease in enzyme activity could be explained through phosphate inhibition. The enzyme was possibly inhibited also by some other phosphate compound(s). Apparently the decrease was not due to instability of the enzyme: a 100-fold dilution of the aged concentrated extracts and storage of the dilutions at $+4°$ for 20 hr did not only restore the enzyme activities

but increased them, at the early developmental stages, above the values obtained in the diluted fresh extracts. The same increase occurred in preparations kept at −20° for 20 hr before dilution. The effect was about 2-fold at the amoeba stage and very low at the sorocarp stage (Fig. 7). The nature of this increase was not studied. It might, however, have involved an activation or release of preformed enzyme protein. Increase in alkaline phosphatase activity *in vitro* has been reported for *Bacillus subtilis* (Whiteley & Oishi, 1963).

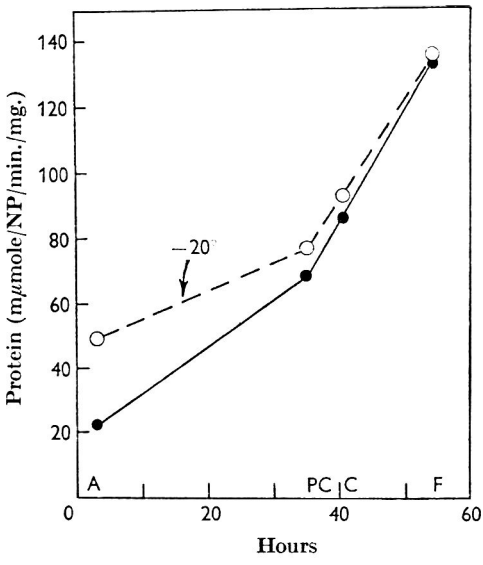

Fig. 7

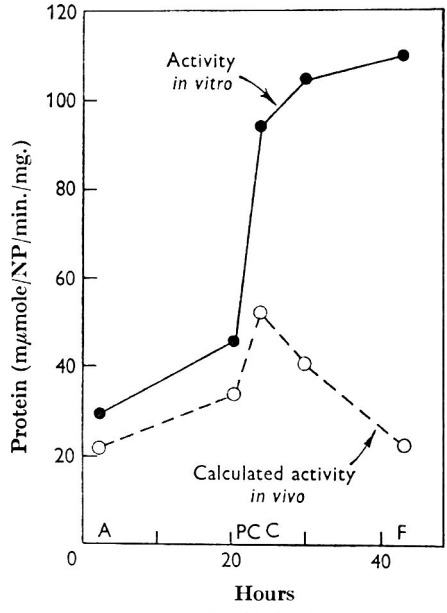

Fig. 8

Fig. 7. Alkaline phosphatase activity *in vitro* during differentiation. The lower curve represents the activity in the fresh extracts. The upper curve shows the release of enzyme activity obtained through freezing. The activities were assayed in 0·5 M-tris without $MgCl_2$. The symbols along the abscissa in this and subsequent graphs refer to amoeba (A), pre-culmination (PC), culmination (C), and sorocarp (F) stages.

Fig. 8. Specific phosphatase activity against NPP during development. The calculated activity *in vivo* is based on the amount of orthophosphate present in the cells at the various stages of differentiation. The extracts were assayed in 0·05 M-tris, and 0·1 M-$MgCl_2$.

Phosphatase activity in vitro and in vivo

Figure 8 shows both the alkaline phosphatase activities *in vitro* and the predicted enzyme activities *in vivo* based on the intracellular phosphate molarities and assuming that the inhibitor was available to the enzyme. The data indicate an increase during differentiation in enzyme concentration but suggest that the inhibition of the enzyme *in vivo* resulted in highest activity during culmination.

Alkaline phosphatase in cells differentiating in the presence of exogenous metabolites

In all the previous experiments the slime mould was differentiating on plain 2% agar in the absence of any exogenous nutrients. When orthophosphate or

glucose was present in the agar, the rate of differentiation was increased. The optimal stimulating concentrations were 0·01 M with respect to Pi and 0·05 M for glucose (Krichevsky & Wright, 1963). In the presence of 0·01 M Pi the intracellular Pi values were higher than when the slime mould was differentiating on plain agar, but only early in development. The high Pi concentrations in the sorocarps were not further increased (Table 9). On plain agar, the intracellular glucose content was highest during pre-culmination and culmination (Wright, Brühmüller & Ward, 1964). Although direct determinations were not made, it is known that exogenous glucose does enter the cells during differentiation, since it stimulates the rate of development and respiration and also affects the pathways of hexose metabolism (Wright *et al.* 1964).

Table 9. *Pi molarity* in cells differentiating on plain agar and on agar* 0·01 M *with respect to Pi*

Stage	Molarity Pi*	
	Plain agar	0·01 M Pi agar
Aggregation	0·002	0·005
Pseudoplasmodium	0·002	0·005
Culmination	0·005	—
Very young sorocarp†	—	0·011
1 day sorocarp	0·013	0·013
2 day sorocarp	0·021	0·018

* Expressed in terms of packed cell volume.
† The stalks were included in all the sorocarp preparations.

Figure 9 shows the alkaline phosphatase activity *in vitro* from cells differentiating on plain agar, and on agar 0·01 M with respect to Pi or 0·05 M with respect to glucose. The enzyme was assayed, as always, under conditions of no Pi inhibition. In the presence of exogenous Pi or glucose, the phosphatase values did not increase after culmination, while in the control the enzyme concentration was maximal in the sorocarps. Early in development there was no negative effect of either Pi or glucose. On the contrary, the phosphatase concentration was higher in the cells on Pi agar. This is most likely due to the fact that phosphate is speeding up the rate of development.

No attempts were made to study the synthesis of the alkaline phosphatase protein. However, the data in Fig. 9 suggest that the enzyme was repressed, but only in the sorocarps, in the presence of glucose or Pi. This effect of Pi, and probably also of glucose, appeared to be a delayed phenomenon, since the alkaline phosphatase levels were not affected in the early stages, though the intracellular Pi concentration was increased when the compound was present in the agar (Table 9). This was more strikingly demonstrated when 0·1 M Pi was used. In an experiment with this concentration, the Pi inside the cells was 0·028 M in the young pre-culmination stage as compared to 0·002 M in the somewhat slower control (still pseudoplasmodia). There was thus a 14-fold increase in intracellular Pi, yet the alkaline phosphatase concentration was not affected in the early stages of development.

The SM medium contained, in addition to potassium phosphate (0·016 M) and glucose (0·05 M), peptone and yeast extract. Excluding the yeast extract, this was

the substrate used in the investigations by Bonner *et al.* (1955), Krivanek (1956) and Krivanek & Krivanek (1958). On this medium the effect on the enzyme was much more pronounced than in the presence of Pi or glucose alone; the phosphatase concentration decreased drastically in the sorocarps (Fig. 10). When concentrated extracts were used (as in the experiments by Krivanek, 1956), the phosphatase values obtained for the sorocarps were still lower, due to phosphate inhibition in the extracts.

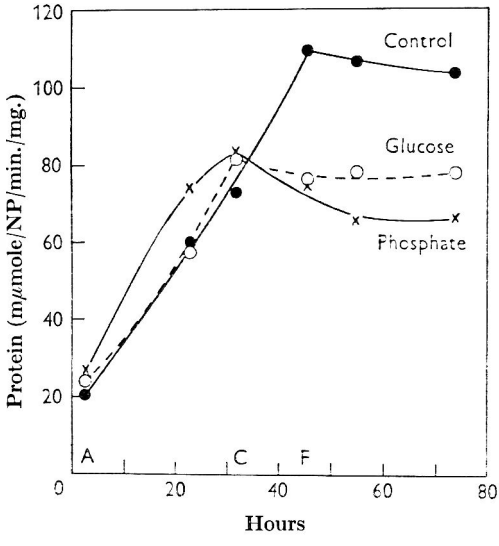

Fig. 9

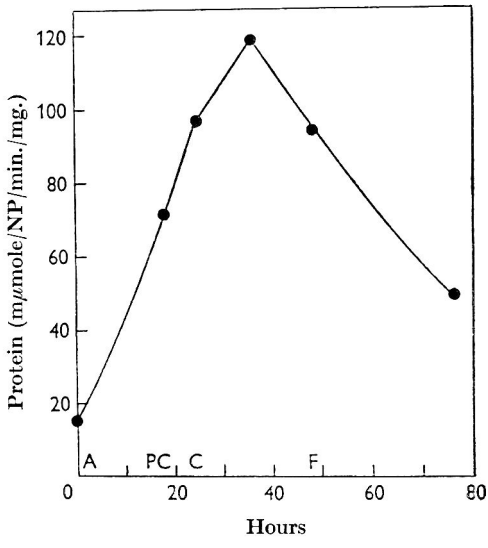

Fig. 10

Fig. 9. Specific phosphatase activity against NPP during development for cells differentiating on plain 2% agar without any supplements (control), on 2% agar, 0·01 M-potassium phosphate and on 2% agar, 0·05 M-glucose. The activities of the diluted, dialysed extracts were assayed in 0·05 M-tris, 0·02 M-$MgCl_2$.

Fig. 10. Specific phosphatase activity against NPP during development for cells differentiating on the SM medium in the presence of residual food bacteria. The diluted, dialysed extracts were assayed in 0·05 M-tris, 0·02 M-$MgCl_2$.

Table 10. *Specific enzyme activities in extracts from sorocarps formed in the presence of exogenous adenosine and deoxyadenosine*

	Specific enzyme activities				
		Adenosine		Deoxyadenosine	
Stage	Control	5×10^{-4} M	5×10^{-3} M	5×10^{-4} M	5×10^{-3} M
Very young sorocarp	184	174	120	194	200
2-day sorocarp	180	300	308	310	400

The diluted, dialysed extracts were assayed in 0·05 M-tris, 0·02 M with respect to $MgCl_2$.

The nucleosides adenosine and deoxyadenosine, end products of the 5′-nucleotidase reaction, were somewhat inhibitory to the rate of slime mould development. The inhibition in the presence of adenosine was quite obvious at 5×10^{-3} M. As shown in Fig. 11, both compounds increased the specific enzyme activities in the

sorocarps and they seemed thus to induce alkaline phosphatase formation in Dictyostelium. The data in Table 10 suggest that deoxyadenosine had a stronger effect than adenosine. All extracts were assayed after dilution and dialysis, i.e. in the absence of any removable inhibitor.

Figure 12, bottom, shows the Pi concentrations in cells differentiating on plain agar and on agar 5×10^{-3} M in respect to adenosine. Early in development the intracellular Pi molarities were the same in both cases, but during sorocarp formation and in the sorocarps the Pi concentration was considerably lower in the presence of

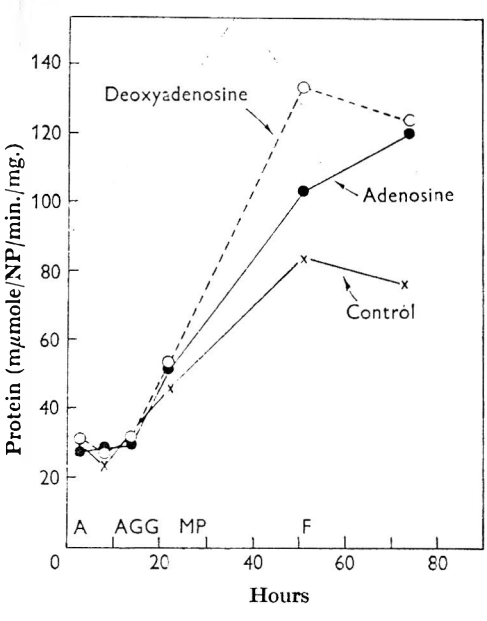

Fig. 11

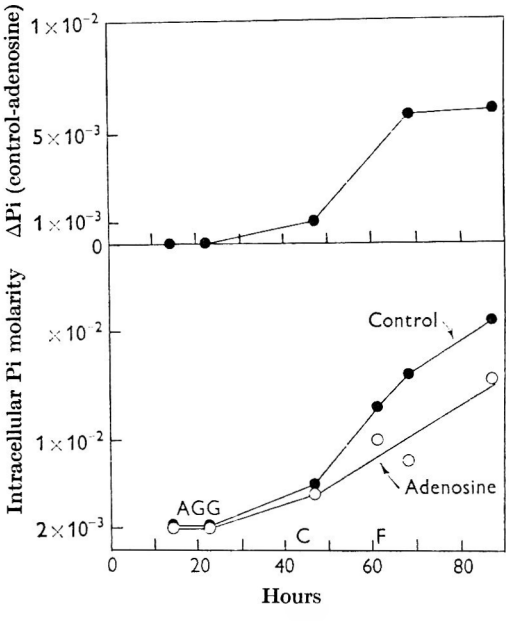

Fig. 12

Fig. 11. Specific phosphatase activity against NPP for cells differentiating on plain 2% agar (control) or agar 5×10^{-3} M in adenosine and deoxyadenosine respectively. The diluted dialysed extracts were assayed in 0·05 M-tris, 0·02 M-$MgCl_2$. The symbols indicate amoeba (A), aggregation (AGG), migrating pseudoplasmodium (MP) and sorocarp (F) stages.

Fig. 12. Lower graph: Intracellular Pi molarity (expressed in terms of packed cell volume) during development on 2% plain agar (control) and on agar 5×10^{-3} M-adenosine. Top graph: Plot of the calculated difference in intracellular Pi molarities between cells on plain and adenosine agar during development.

exogenous adenosine. The upper part of Fig. 12 represents a plot of the calculated difference in intracellular Pi between cells on plain and on adenosine agar. The resulting curve rises during sorocarp formation and in the young sorocarps, the stage at which the stimulation of alkaline phosphatase levels occurred in the presence of adenosine.

DISCUSSION

Of naturally occurring compounds the alkaline phosphatase of *Dictyostelium discoideum*, routinely assayed with NPP, hydrolyses AMP and dAMP. It is in this respect completely different from the unspecific alkaline phosphatase of *Escherichia*

coli which hydrolyses mononucleotides and hexosephosphates (Torriani, 1960; Garen & Levinthal, 1960) and such compounds as ATP, ADP and PPi (Heppel, Harkness & Hilmoe, 1962). The specific 5′-nucleotidases studied usually attack several mononucleotides but not NPP (Reis, 1951; Heppel & Hilmoe, 1951; Sulkowski, Björk & Laskowski, 1963).

The *in vitro* activity pattern obtained on plain agar for βGP, a very poor substrate for the Dictyostelium enzyme, resembled that for NPP. In the presence of nutrients, the activity pattern for NPP (Fig. 10) was the same as that shown for βGP (Bonner *et al.* 1955; Krivanek, 1956). A comparison between the Krivanek's data for alkaline phosphatase (1956) and for 5′-nucleotidase (1958) shows identical distribution of the enzymes. We consider it most probable that these enzymes are one and the same, and that the enzyme studied by us with NPP and 5′-AMP as substrates is identical with the alkaline phosphatase described by Bonner *et al.* (1955) and by Krivanek (1956).

The alkaline phosphatase in Dictyostelium is inhibited by orthophosphate. Data (Fig. 9) suggest that the enzyme is also repressed by Pi, but only in sorocarps. In *Escherichia coli* the alkaline phosphatase is both inhibited and repressed by Pi (Horiuchi, Horiuchi & Mizuno, 1959; Torriani, 1960). Glucose, a known repressor of many enzymes (Neidhart & Magasanik, 1956) causes the same decrease in enzyme concentration in the spores as Pi. The rich medium, which contains glucose, Pi and peptone, is more powerful than glucose or Pi alone. Whether this depends on the combination of Pi and glucose or in part on the presence of some amino acid(s) in peptone (Cox & MacLeod, 1963) is not known.

The alkaline phosphatase activity *in vivo* in *Dictyostelium discoideum* appears to be controlled by at least two systems: (1) End-product inhibition by Pi. This is perhaps most important when the organism differentiates under extreme conditions of starvation. (2) Repression. The accumulation of the enzyme in the sorocarps is inhibited by the presence of exogenous metabolites in the environment during development. Both systems appear to collaborate to ensure maximal alkaline phosphatase activity during culmination, i.e. when cellulose synthesis and the final differentiation into spores and stalk cells takes place. The conclusions are based on the assumption that the Pi concentration determined for the whole cell approximates the concentration for the enzyme in the cell. The significance of *in vitro* data with respect to *in vivo* conditions has been discussed elsewhere (Wright, 1964).

In the presence of exogenous adenosine or deoxyadenosine the alkaline phosphatase concentrations increased, but only in the sorocarps (Fig. 11). If this effect involves increased enzyme synthesis, considerable protein synthesis must occur in the spores. That this is the case was shown by Wright & Anderson (1960).

The effect of the nucleosides could be due to one or both of the following mechanisms: (1) The inducer may not be adenosine or deoxyadenosine *per se* but AMP or dAMP formed inside the cells. (2) Another possibility is that the increase in enzyme concentration is due to the removal of the repressor, Pi, by the formation of AMP or dAMP from the base + Pi. We cannot at present decide between these two interpretations. We can only point out that the time of increase in alkaline phosphatase in the presence of adenosine coincides with the time of decrease in intracellular Pi concentration.

A preliminary report of this work has been published, (Gezelius & Wright, 1963).

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REFERENCES

BONNER, J. T. (1959). *The Cellular Slime Molds.* Princeton, N.J.: Princeton University Press.

BONNER, J. D., CHIQUOINE, A. D. & KOLDERIE, M. Q. (1955). A histochemical study of differentiation in the cellular slime molds. *J. expt. Zool.* **130**, 133.

COX, R. P. & MACLEOD, C. M. (1963). Repression of alkaline phosphatase in human cell cultures by cystine and cysteine. *Proc. natn. Acad. Sci., U.S.A.* **49**, 504.

DRYER, R. L., TAMMES, A. R. & ROUTH, J. I. (1957). The determination of phosphorus and phosphatase with *N*-phenyl-*p*-phenylenediamine. *J. biol. Chem.* **225**, 177.

EADIE, G. S. (1952). On the evaluation of the constants V_m and K_m in enzyme reactions. *Science*, **116**, 688.

FISKE, G. H. & SUBBAROW, Y. (1925). The colorimetric determinations of phosphorus. *J. biol. Chem.* **66**, 375.

GAREN, A. & LEVINTHAL, C. (1960). A fine-structure genetic and chemical study of the enzyme alkaline phosphatase of *E. coli*. *Biochim. biophys. Acta*, **38**, 470.

GEZELIUS, K. & WRIGHT, B. (1963). Alkaline phosphatase and inorganic phosphate in *Dictyostelium discoideum*. *Bact. Proc.* p. 68.

HEPPEL, L. A., HARKNESS, D. R. & HILMOE, R. J. (1962). A study of the substrate specificity and other properties of the alkaline phosphatase of *Escherichia coli*. *J. biol. Chem.* **237**, 841.

HEPPEL, L. A. & HILMOE, R. J. (1951). Purification and properties of 5′-nucleotidase. *J. biol. Chem.* **188**, 665.

HERMAN, E. C. & WRIGHT, B. E. (1959). A 5′-nucleotidase activated by ferrous iron. *J. biol. Chem.* **234**, 122.

HORIUCHI, T., HORIUCHI, S. & MIZUNO, D. (1959). A possible negative feedback phenomenon controlling formation of alkaline phosphomonoesterase in *Escherichia coli*. *Nature, Lond.* **183**, 1529.

KORNBERG, A. & PRICER, W. E. (1951). Enzymic phosphorylation of adenosine and 2,6-diaminopurine riboside. *J. biol. Chem.* **193**, 481.

KRICHEVSKY, M. I. & WRIGHT, B. E. (1963). Environmental control of the course of development in *Dictyostelium discoideum*. *J. gen. Microbiol.* **32**, 195.

KRIVANEK, J. O. (1956). Alkaline phosphatase activity in the developing slime mold, *Dictyostelium discoideum* Raper. *J. exp. Zool.* **133**, 459.

KRIVANEK, J. O. & KRIVANEK, R. C. (1958). The histochemical localization of certain biochemical intermediates and enzymes in the developing slime mold, *Dictyostelium discoideum* Raper. *J. exp. Zool.* **137**, 89.

LIDDEL, G. U. & WRIGHT, B. E. (1961). The effect of glucose on respiration of the differentiating slime mold. *Develop. Biol.* **3**, 265.

NEIDHARDT, F. C. & MAGASANIK, B. (1956). The effect of glucose on the induced biosynthesis of bacterial enzymes in the presence and absence of inducing agents. *Biochim. biophys. Acta*, **21**, 324.

RAPER, K. B. (1951). Isolation, cultivation and conservation of simple slime molds. *Quart. Rev. Biol.* **26**, 169.

REIS, J. L. (1951). The specificity of phosphomonoesterases in human tissues. *Biochem. J.* **48**, 548.

SCHWARTZ, J. H. (1963). The phosphorylation of alkaline phosphatase. *Proc. natn. Acad. Sci., U.S.A.* **49**, 871.

SULKOWSKI, E., BJÖRK, W. & LASKOWSKI, M., SR. (1963). A specific and non-specific alkaline monophosphatase in the venom of *Bothrops atrox* and their occurrence in the purified venom phosphodiesterase. *J. biol. Chem.* **238**, 2477.

SUSSMAN, M. (1961). Cultivation and serial transfer of the slime mould *Dictyostelium discoideum* in liquid nutrient medium. *J. gen. Microbiol.* **25**, 375.

TORRIANI, A. (1960). Influence of inorganic phosphate in the formation of phosphatases by *Escherichia coli*. *Biochim. biophys. Acta*, **38**, 460.

WHITELEY, H. R. & OISHI, M. (1963). An increase in alkaline phosphatase in an *in vitro* system derived from *Bacillus subtilis*. *Biochem. biophys. Res. Comm.* **13**, 6.

WRIGHT, B. E. (1960). On enzyme-substrate relationships during biochemical differentiation. *Proc. natn. Acad. Sci., U.S.A.* **46**, 798.

WRIGHT, B. E. (1964). Biochemistry of Acrasiales. In *Biochemistry and Physiology of Protozoa*. Ed. by S. Hutner, vol. 3. New York: Academic Press.

WRIGHT, B. E. & ANDERSON, M. L. (1960). Protein and amino acid turnover during differentiation in the slime mold. II. Incorporation of [^{35}S]methionine into the amino acid pool and into protein. *Biochim. biophys. Acta*, **43**, 67.

WRIGHT, B. E. & BARD, S. (1963). Glutamate oxidation in the differentiating slime mold. I. Studies *in vivo*. *Biochim. biophys. Acta*, **71**, 45.

WRIGHT, B. E., BRÜHMÜLLER, M. & WARD, C. (1964). Studies *in vivo* on hexose metabolism in *Dictyostelium discoideum*. *Develop. Biol.* **9**, 287.

ZAMENHOF, S. (1957). Preparation and assay of deoxyribonucleic acid from animal tissue. *Meth. Enzymol.* **3**, 696.

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The Identification of Atypical Strains of *Pseudomonas aeruginosa*

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SUMMARY

The diversity of colonial types of *Pseudomonas aeruginosa* which may be encountered is described, together with a series of biochemical tests and the application of serological and pyocine typing which are of use in identifying atypical strains. These methods are particularly recommended for strains which do not form pigment. It is suggested that absence of pigment production even on special media does not preclude the possibility that a strain is *P. aeruginosa*.

INTRODUCTION

Members of the genus *Pseudomonas* are widely distributed in nature, but the only common human pathogen is *Pseudomonas aeruginosa*. In the clinical laboratory, therefore, the practical problem is to distinguish *P. aeruginosa* from other Pseudomonas species. The identification of typical strains of *P. aeruginosa* presents no difficulty, but the variability of the characteristics of this organism was recognized even in early work, the occurrence of non-pigment forming strains being particularly emphasized (Blanc, 1923; Cataliotti, 1935); Gaby & Free (1953) stressed the importance of recognizing such strains in clinical material. These now appear to be occurring more frequently in hospital practice (Darrell & Wahba, 1964). It has been suggested that the use of antibiotics may favour their occurrence (Seligmann & Wassermann, 1947; Fujita, 1957; Schneierson, Amsterdam & Perlman, 1960). Whether the increase in atypical strains is a true one, or merely results from an increasing interest in the group, their importance is not limited to clinical material and workers in many fields are faced with the problem of identifying such strains. Many techniques have been described for enhancing pigment production (Turfitt, 1936; Burton, Campbell & Eagles, 1948; King, Ward & Raney, 1954; Lutz, Schaeffer & Hofferer, 1958; Frank & De Moss, 1959; Rosenfeld & Appel, 1963), but none has been widely used, mainly because of practical limitations on the number of different media routinely used in any laboratory. Pigment formation is usually not obvious on the blood agar used in clinical laboratories for primary cultures, and after overnight incubation may be absent. In practice, identification of *P. aeruginosa*

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is based initially on the characteristic colonial appearance and the typical smell. The variability of the former, in particular, is not generally recognized, and often identification is carried no further. In the present work, 1961 Pseudomonas strains from various sources were examined (Wahba, 1964*a*). A series of tests was developed, applied to 250 typical *P. aeruginosa* strains (producing pyocyanin and smelling of trimethylamine) and found to give uniformly positive results. These tests were then applied to all *P. aeruginosa* from human sources not producing pyocyanin, provisionally identified by a positive oxidase test and oxidative utilization of glucose in Hugh & Leifson's medium, and also to a group of miscellaneous Pseudomonas strains from various sources. Six colonial variants were encountered.

METHODS

Organisms. The colonial appearances of 1961 Pseudomonas strains submitted for typing, or obtained from type culture collections and other sources, were examined; these were provisionally accepted as such on the grounds that they were oxidase-positive, motile, Gram-negative rods with oxidative use of glucose in Hugh & Leifson's medium. Among them 1899 cultures were identified as *Pseudomonas aeruginosa* on the grounds either of being typical (producing pyocyanin and smell) or, when possessing atypical characters, by means of the series of tests described below. The following 272 Pseudomonas strains were selected for the present study. (*a*) Six strains, each representing one of the different colonial types of *P. aeruginosa*, (*b*) 185 strains from human sources which did not produce visible pigment on stock nutrient agar after incubation at 37° for 24 hr and 2 days at room temperature. (*c*) A miscellaneous group made up as follows: (i) 9 strains of *P. aeruginosa* from various non-human sources (including a locust and a sample of barrier cream); (ii) 10 strains originally submitted under names other than *P. aeruginosa*, but thought as a result of our tests to be *P. aeruginosa*; (iii) 20 strains from human infections or environment submitted as *P. aeruginosa* but found on testing to be other species of Pseudomonas and not further identified; (iv) 42 strains of Pseudomonas other than *P. aeruginosa*.

Media. Stock nutrient agar No. 1 (Oxoid No. 1); Lab Lemco beef extract, 1 g.; yeast extract (Oxoid L 20), 2 g.; peptone (Oxoid L 37), 5 g.; sodium chloride, 5 g.; agar, 15 g.; distilled water to 1 l.; pH 7·4.

Nutrient agar No. 2 (Oxoid); Lab Lemco beef extract, 10 g.; peptone (Oxoid L 37), 10 g.; sodium chloride, 5 g.; 'Ionagar' No. 2, 10 g.; distilled water to 1 l.; pH 7·4.

Details of other media are given under techniques.

Techniques

Oxidase test. Rogers' (1963) modification of Kovàcs' (1956) method. Strains not growing at 37° were incubated at 22°.

Motility. By microscopy of hanging drop.

Oxidation versus fermentation test of Hugh & Leifson (1953).

Growth on nutrient agar No. 1 at 42° for three consecutive subcultures (Haynes, 1951, 1962). Slopes were incubated in a covered tin immersed in a 43° water bath, thus ensuring that the actual temperature inside the culture tubes, as recorded

by a thermometer in a blank tube, was in fact 42°. Control cultures were also incubated at 37° and 22°.

Oxidation of potassium gluconate and production of slime within 3 days at 37° and 42°. A modification of Haynes' (1951) original technique was used. The medium was dispensed in 5 ml. amounts and every strain was inoculated in duplicate, one tube being incubated at 37° and the other at 42° (to investigate the possibility of combining three tests for identifying *Pseudomonas aeruginosa*). The cultures were not shaken during incubation. After incubation for 48 hr all tubes were examined for slime production by shaking. When slime was present, the cultures were then tested for reducing substances by adding half a 'Clinitest' tablet (Ames Co., Stoke Poges, Bucks, used for the estimation of sugar in urine) (Carpenter, 1961). When slime was absent, the culture was divided; one half was tested for the presence of reducing substance and the other incubated for a further 5 days at the appropriate temperature and re-examined. Strains not growing at 37° were incubated at 22°.

Growth on 1% triphenyltetrazolium chloride in nutrient agar No. 1 (Selenka, 1958). 16 strains were inoculated per plate, the source of the inoculum being nutrient agar No. 1 slopes.

Growth on 0·2% (w/v) cadmium sulphate in nutrient agar No. 1. This medium was modified from one containing cadmium chloride devised by Shkeir, Losse & Bahn (1963).

Production of gelatinase by the plate method of Clarke (1953) with nutrient agar No. 1 as base and inoculating 16 strains per plate. Results read after 24 hr. Test repeated with negative strains and read after 3 days.

Production of collagenase. This was tested by a modification of the method devised by Oakley, Warrack & van Heyningen (1946). Layered nutrient agar No. 1 plates were prepared, the upper layer containing 0·5% azocoll (hide powder coupled to a red azo dye). Sixteen strains were inoculated on each plate, incubated for 3 days and observed daily. The test was later performed by spotting melted azocoll-containing nutrient agar No. 1 on a basal layer of 2% agar in water and inoculating the solidified islets of medium with the strains.

Enhancement of pyocyanin production on a medium modified from Sierra (1957) and containing (g.): Bactopeptone, 10; sodium chloride, 5; $CaCl_2H_2O$, 0·2; $MgSO_4.7H_2O$, 0·1; agar, 18; distilled water to 1 l. Tween 80 solution, autoclaved separately, was added in a final concentration of 1%; pH 7·4. For comparative purposes, strains were also inoculated on nutrient agar No. 2 plates, as this medium improved the production of pyocyanin in some strains.

Production of pyocines (Hamon, 1956). This test apart from providing a means of typing *Pseudomonas aeruginosa*, appeared to give good results as a diagnostic procedure. Details of the procedure have been published (Wahba & Lidwell, 1963; Darrell & Wahba, 1964); briefly it consists of cross-streaking 12 indicator strains at right angles to the strain to be tested which had been previously killed with chloroform vapour and removed with a microscope slide.

Slide-agglutination with a polyvalent Pseudomonas aeruginosa antiserum. This was made by mixing 14 monospecific sera (Wahba, 1964*b*). Individual sera were produced in rabbits using 14 different serotypes; these included 12 types described by Habs (1957), type 13 (Sandvik, 1960) and type 14 (Wahba, 1964*a*). Saline suspensions made from overnight agar cultures were boiled for 2½ hr and injected into

Table 1. *Pseudomonas aeruginosa—Colonial types*

Descriptions appl y to colonies on nutrient agar after 24 hr incubation at 37° except for Type 6 which required 48 hr incubation to produce visible growth

Type	Size (mm.)	Shape	Elevation	Surface	Edge	Production of visible pigment	Opacity	Consistency	Emulsifiability	Suspension	Description by
1. 'S'*	2–3	Irregular	Effuse	Smooth matt not rough	Fimbriate with lobate edge	Variable characteristically blue-green	Semi-opaque	Butyrous	Easy	Uniform	Gaby (1955)
2. 'SR'	1–2	Circular	Convex	Smooth, shining	Entire	Variable	Translucent	Slightly viscid	Easy	Uniform	Köhler (1958)
3. 'R'*	2–3	Irregular	Raised with umbonate centre	Rough, matt	Undulate	Variable	Opaque	Butyrous	Difficult	Granular	Gaby (1955) Köhler (1958)
4. Mucoid	1–3	Circular, tend to coalesce	Convex	Smooth, shining	Entire	Usually none, colony yellowish-white	Almost transparent	Wet and viscid	Difficult	Streaky	Sonnenschein (1927) Schwarz & Lazarus (1947) Henriksen (1948) Munoz & Dougherty (1951)
5. Gelatinous*	2–3	Circular	Raised with higher centre	Smooth, shining	Entire	Variable	Opaque	Membranous or jelly-like	Difficult	Stringy	Schultz (1947) Danz & Schultz (1949)
6. Dwarf	0·5 or less	Circular	Convex	Smooth, shining	Entire	Variable	Translucent	Slightly viscid	Easy	Uniform	Wahba (1964*a*)

* Only types marked thus show metallic sheen.

rabbits in increasing volumes (from 0·1 to 2 ml.) on alternate days; the initial dose was given subcutaneously and the remainder intravenously in the ear vein. Strains for agglutination tests were taken from young cultures on nutrient agar plates or slopes (incubation 16 hr or less). When the strain appeared to be autoagglutinable or mucoid it was plated on other media, especially pyocine-typing medium (Wahba, 1963), which decreased the formation of mucoid substance.

RESULTS

Colonial morphology. Six colonial types of *Pseudomonas aeruginosa* were observed. Their main features are summarized in Table 1, which applies to colonies on nutrient agar No. 1 after incubation for 24 hr at 37°, except for type 6 (dwarf) where the period of incubation was 48 hr. Typical examples of the six types are shown in Plate 1 (figs. 1–6); Table 2 gives the incidence of the various types among 1090 human strains.

The classical metallic sheen (Wahba, 1964*c*) was seen only with types 1, 3 and 5; it may occur as small 'plaques', not to be confused with phage plaques (Warner, 1950). Mucoid colonies rapidly lose water and flatten on the medium giving a contoured or map-like appearance. There is considerable instability of colonial form on subculture; this is particularly marked with the gelatinous type (Schultz, 1947) which tends to lose many characteristics, but retains its firm adherence to the medium.

Table 2. *Incidence of various colonial forms in* 1090 *strains of Pseudomonas aeruginosa from human material*

	%	
(*a*) Mixed S and SR, or S and R, or S, SR and R, or S and dwarf	81·3	(the majority showing a predominantly S type of colony)
(*b*) R form only	16·4	
(*c*) Mucoid form only	1·2	
(*d*) Gelatinous form only	0·4	
(*e*) Dwarf form only	0·7	

Growth requirements and biochemical reactions. The results obtained with the 185 *Pseudomonas aeruginosa* strains not forming pyocyanin on nutrient agar No. 1 and isolated from human sources were all uniform and corresponded to the pattern of the first strain in Table 3 (i) which was also the pattern of typical pyocyanin- or pyorubin-producing strains examined in the preliminary stages of this work. The results obtained with the miscellaneous group of organisms are shown in Table 3 (i–iv). The performance in the various tests of the strains grouped according to their final identification is given in Table 4.

Growth at 42°. Results given in Table 3 (i–iv) are for a single subculture as this is the usual routine in diagnostic laboratories. Although the test has been considered to be one of the most reliable features distinguishing *Pseudomonas aeruginosa* from other Pseudomonas species over 50% of the latter in our series were able to grow at this temperature on one occasion. They did not survive three subcultures (Haynes, 1962) but such a procedure would not be a practical routine test.

Table 3. *Results of eleven tests applied to the group of miscellaneous Pseudomonas strains (group c).*

+ = good growth or positive result of the test. ± = scanty growth or weakly positive test. − = no growth or negative result of the test. With strain showing scanty or no growth at 37°, gluconate oxidation, gelatinase, collagenase tests and pyocine typing were done at room temperature.

Strains received as	Source	Growth at 42°	Growth at 37°	Oxidation of gluconate and slime	Growth on tetrazolium	Growth on cadmium sulphate	Gelatinase	Collagenase	Pigment on special medium	U.v. fluorescence	Typability by pyocine	Typability by serology	Final diagnosis
(i) *P. aeruginosa* from unusual sources													
P. aeruginosa	Barrier cream	+	+	+	+	+	+	+	+	+	O	9	*P. aeruginosa*
	Water	+	+	±	+	+	+	+	+	+	D	auto	
	Soil	+	+	+	+	+	+	+	−	−	NT	1	
	Plant	+	+	+	+	+	+	±	+	+	D	6	
	Plant	+	+	+	+	+	+	+	+	+	F	11	
	Water	+	+	+	+	+	+	+	+	+	NT	1	
	?	+	+	+	+	+	+	±	+	+	B	2ab	
	Locust	±	+	±	+	+	+	±	+	+	NT	1	
	Sink	+	+	+	±	±	+	−	+	+	NT	3	
(ii) *Pseudomonas* now thought to be *P. aeruginosa*													
Pseudomonas	?	+	+	+	+	+	+	+	+	+	A	10	*P. aeruginosa*
	?	+	+	+	+	+	+	+	+	+	NT	1	
	Sea	+	+	+	±	−	+	+	+	+	D	6	
	Milk	+	+	+	±	+	+	+	+	+	NT	3	
	?	+	−	−	−	+	+	+	+	+	D	6	
P. fluorescens	?	+	+	+	+	+	+	+	+	+	NT	1	
P. ovalis	Sea	+	+	+	±	−	−	−	+	+	NT	1	
P. polycolor	Lettuce	+	+	+	+	+	+	+	+	+	F	11	
	Plant	+	+	+	±	+	+	+	+	+	A	2ab	
	Plant	+	+	+	+	+	+	−	+	+	D	6	

NT = not typable. auto = autogglutinable.

Table 3 (*cont.*)

(iii) Organisms from human infections and the human environment, submitted as *Pseudomonas aeruginosa*

Strains received as *P. aeruginosa*	Source	Growth at 42°	Growth at 37°	Oxidation of gluconate and slime	Growth on tetrazolium	Growth on cadmium sulphate	Gelatinase	Collagenase	Pigment on special medium	U.v. fluorescence	Typability by pyocine	Typability by serology	Final diagnosis
	Urine	+	+	−	−	−	−	−	−	+	NT	−	*P. fluorescens*
	Urine	+	+	−	−	−	+	+|	−	+	NT	−	
	Dust	+	+	−	−	−	−	−	−	+	NT	−	
	Dust	+|	+	−	−	−	−	−	−	+	NT	−	
	Sink	+|	+	−	−	−	−	−	−	+	NT	−	
	Dust	+|	+	−	−	−	−	−	−	−	NT	−	*Pseudomonas* sp.
	Dust	+	+	−	−	−	−	−	−	−	NT	−	
	Faeces	+	+	−	−	−	−	−	−	−	NT	−	
	Wound swab	+	+	−	−	−	−	−	−	−	NT	−	
	Faeces	+	+	−	−	+	−	−	−	+	NT	−	*P. fluorescens*
	Dust	−	+	−	−	−	−	−	−	+	NT	−	
	Urine	−	+	−	−	−	−	−	−	+	NT	−	
	Faeces	+|	+	−	−	−	−	−	−	+	NT	−	
	Sink	+	+	−	−	−	−	−	−	−	NT	−	*Pseudomonas* sp.
	Dust	+|	+	−	−	−	+	−	−	+	NT	−	*P. fluorescens*
	Dust	+	+	−	−	−	−	−	−	+	NT	−	
	Faeces	+	+	−	−	−	+	−	−	−	NT	−	*Pseudomonas* sp.
	Dust	−	+	−	−	−	−	−	−	+	NT	−	*P. fluorescens*
	Ear	+	+	−	−	−	+	−	−	+	NT	−	
	Urine	+	+	−	−	+	−	−	−	−	NT	−	*Pseudomonas* sp.

Table 3 (*cont.*)

(iv) Various other than *P. aeruginosa*

Strains received as	Source	Growth at 42°	Growth at 37°	Oxidation of gluconate and slime	Growth on tetrazolium	Growth on cadmium sulphate	Gelatinase	Collagenase	Pigment on special medium	U.v. fluorescence	Typability by pyocine	Typability by serology	Final diagnosis
Pseudomonas	Sputum	+/−	+	−	−	−	−	−	−	−	NT	−	*Pseudomonas* sp.
	Sputum	−	+	−	−	−	−	−	−	−	NT	−	
	Sputum	−	−	−	−	−	−	−	−	−	NT	−	
	Wound	+	+	−	−	−	−	−	−	−	NT	−	
	Wound	+	+	−	−	−	−	−	−	−	NT	−	
	Urine	−	−	−	−	−	−	−	−	−	NT	−	
	Burn	+	+	−	−	−	−	−	−	−	NT	−	
	Burn	−	−	−	−	−	+/−	−	−	−	NT	−	
	Burn	+	+	−	−	−	+	−	−	−	NT	−	
	Burn	+	+	−	−	−	+	−	−	−	NT	−	
	Sink	+	+	−	−	−	−	−	−	−	NT	−	
	Sink	+	+	−	−	−	−	−	−	−	NT	−	
	Water	−	−	−	−	−	−	−	−	+	NT	−	*P. fluorescens*
	Shellfish	+	+	−	−	−	−	−	−	+	NT	−	
	Fish	+	+	−	−	−	−	−	−	−	NT	−	*Pseudomonas* sp.
	Snake faeces	−	+	−	−	−	−	−	−	−	NT	−	
P. fluorescens	Cerebro-spinal fl.	−	−	−	−	−	+	−	−	+	NT	−	*P. fluorescens*
	Cerebro-spinal fl.	−	+/−	+/−	−	−	+/−	−	−	+	NT	−	
	Water	+/−	+/−	+/−	+/−	−	−	−	−	−	NT	−	*Pseudomonas* sp.
	Plant	−	−	−	−	−	+	−	−	−	NT	−	*P. fluorescens*
	Plant	−	−	−	−	−	+	−	−	+	NT	−	
P. ovalis	Urine	+	+	−	−	−	−	−	−	−	NT	−	*Pseudomonas* sp.
P. putida	Soil	+	+	−	−	−	−	−	−	−	NT	−	
	Blood culture	+	+	−	−	−	−	−	−	−	NT	−	

Table 3 (*cont.*)

(iv) (*cont.*)

Strains received as	Source	Growth at 42°	Growth at 37°	Oxidation of gluconate and slime	Growth on tetrazolium	Growth on cadmium sulphate	Gelatinase	Collagenase	Pigment on special medium	U.v. fluorescence	Typability by pyocine	Typability by serology	Final diagnosis
P. septica	Plant	−	−	−	−	−	+	−	−	+	NT	−	*P. fluorescens*
P. synxantha	Plant	−	−	−	−	−	+	−	−	−	NT	−	*Pseudomonas* sp.
P. taetrolens	?	+	+	−	−+	−	+	−+	−	+	NT	−	*P. fluorescens*
P. angulatataboai	Mineral water	−	+	−+	−	−	−+	−	−	−	NT	−	*Pseudomonas* sp.
P. denitrificans	Sea	−	−	−	−	−	−	−	−	−	NT	−	
P. membranoformis	Sea	−	−	−	−	−	−	−	−	−	NT	−	
P. oleovorans	Cutting oil	+	+	−	−	−	−	−	−	−	NT	−	
P. rubescens	Cutting oil	+	+	−	−	−	+	−	−	−	NT	−	
P. cichorii	Plant	−	−	−	−	−	−	−	−	−	NT	−	
	Plant	−	−	−	−	−	−	−	−	−	NT	−	
	Plant	−	−	−	−	−	−	−	−	−	NT	−	
P. syringae	Lilac	−	+	−	−	−	+	−	−	−	NT	−	
	Lilac	−	+	−	−	−	−	−	−	−	NT	−	
P. phaseolicola	Dwarf bean	−	−	−	−	−	−	−	−	−	NT	−	
	Dwarf bean	−	−	−	−	−	−	−	−	−	NT	−	
P. marginalis	Lettuce	−	−	−	−	−	+	−	−	−	NT	−	
	Lettuce	−	−	−	−	−	+	−	−	−	NT	−	
	Lettuce	−	+	−+	−+	−	+	−	−	−	NT	−	

Growth at 37°. While a temperature optimum of less than 37° is a strong indication that a strain is not *Pseudomonas aeruginosa*, the converse is not true. Table 4 shows that a high proportion of our other Pseudomonas cultures (45 of 62) also grew well at this temperature.

Oxidation of gluconate and slime production. This test proved more useful but is time-consuming; four strains other than *Pseudomonas aeruginosa* (see Table 4) gave positive results. Performance of the test at 42° rendered it more specific for *P. aeruginosa.*

Table 4. *Summary of results of eleven tests on the miscellaneous group (c) organisms arranged according to their final identification on the basis of the scheme described*

	Total	Growth at 42°	Growth at 37°	Oxidation of gluconate and slime production	Growth on tetrazolium medium	Growth on cadmium sulphate medium
P. aeruginosa	19	19	19	19	19	18
P. fluorescens	21	12	17	2	2	1
Other Pseudomonas (not further identified)	41	22	28	2	1	1

	Gelatinase	Collagenase	Pyocyanin or pyorubin on special medium	U.v. fluorescence	Typability by pyocine	Typability by serology
P. aeruginosa	19	16	19	19	11	18
P. fluorescens	8	2(±)	—	21	—	—
Other Pseudomonas (not further identified)	12	—	—	—	—	—

Growth in the presence of cadmium sulphate and on tetrazolium media. These media were selective for *Pseudomonas aeruginosa* which, with very few exceptions, produced normal growth on both. Occasionally other Pseudomonas cultures also produced good growth but all other bacterial species were completely inhibited. In addition to its selective properties, cadmium sulphate medium enhanced fluorescin production by *P. aeruginosa.*

Production of proteinases. Gelatinase activity is shared by many other members of the *Pseudomonas* genus (Table 4), and is of little differential importance. Marked collagenase activity is found only in strains of *Pseudomonas aeruginosa*; it was observed twice only among other Pseudomonas cultures and in both the reaction was very weak. A positive reaction is of value in identifying an organism as *P. aeruginosa*, but 3 of 19 strains were negative.

Enhancement of pigment production. One hundred and eighty-five strains (17%) from a total of 1090 *Pseudomonas aeruginosa* cultures isolated from human material did not produce pigment on nutrient agar No. 1 after overnight incubation followed by a period of 2 days at room temperature. Various pigment-enhancing media were tested. Table 5 shows the types of pigment produced by these 185 strains on nutrient agar No. 2 and on the modified Sierra medium finally adopted. Nutrient agar No. 2 which contains more peptone and meat extract than nutrient agar No. 1, gave appreciably better results, but 57 strains still did not show pigment, as against only 34 (3% of the total) on the modified Sierra medium. The latter has the added advantage of stimulating early pigment formation in nearly every case (i.e. after

overnight incubation) whereas with nutrient agar No. 2 an appreciable number of strains still required incubation for 48 hr to show pigment production. When strains produced fluorescent pigment only, this was detectable on this medium under ultraviolet radiation, although no yellow-green colour was apparent to the naked eye.

Ultraviolet fluorescence. The method of Lowbury, Lilly & Wilkins (1962) was used, but as will be seen from Table 3, it did not distinguish *Pseudomonas aeruginosa* from *P. fluorescens*.

Production of pyocines. Production of pyocines against a set of indicator strains provided additional confirmatory evidence that an organism is *Pseudomonas aeruginosa*. In a series of 1090 strains (Darrell & Wahba, 1964), 7·6 % did not produce pyocines. However, strains of *P. aeruginosa* which do not type by both pyocine production and the serological method are very rare.

Use of polyvalent agglutinating serum. Of the series of 1899 strains of *Pseudomonas aeruginosa*, only 8, 4 of which were 'R' forms, did not agglutinate. Cross-agglutination with *P. fluorescens* did not occur with the pooled polyvalent serum, and even with undiluted component sera, cross-agglutination was very infrequent.

Table 5. *Pigment production by strains of Pseudomonas aeruginosa*

Production of pigment within 24 hr at 37° by 185 strains not showing pigment at 24 hr on stock nutrient agar medium.

	Nutrient agar No. 2	Modified Sierra medium
Blue-green	33 (18 %)	66 (35 %)
Yellow-green	83 (45 %)	72 (39 %)
Red or brown	12 (7 %)	13 (7 %)
No pigment*	57 (30 %)	34 (19 %)

* Included here on the strength of their reactions with other tests described.

DISCUSSION

Williamson (1956) stated that a single morphological description for *Pseudomonas aeruginosa* was inadequate. In the present paper, six colonial types are described and it is essential to be aware of this possible range of colonial forms if atypical cultures are to be correctly identified. We have used existing names for types previously described (Gaby, 1955; Köhler, 1958; Schultz, 1947), typical colonies being designated smooth in spite of their matt surface, and the smooth variant, resembling the colonies of Enterobacteriaceae, being called the S-R (smooth-rough) type. Apart from these and the rough, mucoid and gelatinous types previously described, a new form, the dwarf type, is described. It requires 48 hr to produce visible growth and would be missed in many clinical laboratories. Variants commonly occur in one and the same culture (Zierdt & Schmidt, 1964) giving the false impression that different bacterial species are present. Mucoid colonies cause confusion, being usually non-pigmented, and resembling colonies of *Enterobacter aerogenes* and Klebsiella species, from which however, they may be distinguished by the Kovàcs oxidase test. The oxidase reaction is the single most useful screening test for growth from any media. A negative reaction excludes all types of Pseudomonas

encountered in human material (some oxidase-negative species are known, such as *P. maltophila* and some plant pathogens), while an immediate strongly positive reaction is highly suggestive that the organism belongs to this group. Oxidase-positive cultures require confirmatory testing with Hugh & Leifson's medium or for arginase activity (Sherris *et al.* 1959; Taylor & Whitby, 1964), but both these tests establish only that the strain belongs to the genus *Pseudomonas*. The final differentiation of *P. aeruginosa* from other Pseudomonas species is a matter of considerable difficulty. The salient distinguishing feature of the species is the production of pyocyanin or pyorubin and to induce its formation by atypical strains probably remains the most satisfactory method of identifying these. For this purpose, the modified Sierra medium is the most efficient of those tested, with regard to the number of strains which produce pigment and to the rapidity of its appearance. The pigments produced also show a range of colours not fully appreciated in clinical practice in spite of the work by Gessard (1919, 1920).

Agglutination by a polyvalent serum against *Pseudomonas aeruginosa* strains is a very specific test, but of less general application unless large-scale production of the sera became feasible.

In applying the other tests to a selected series of atypical strains, a different pattern of results was obtained with strains in which pigment production could be induced, from the pattern given by the majority of persistently non-pigment-forming strains. Pigment-producing strains grew on cadmium- and tetrazolium-containing media, produced collagenase and in the majority of cases also produced pyocines active against typing strains. They also had a higher temperature optimum and oxidized gluconate with slime production. This pattern of reaction also occurred in a small proportion of persistently non-pigment-forming organisms, constituting 3% of all *Pseudomonas aeruginosa* strains examined. The pattern appears to be specific for *P. aeruginosa* and we believe that such strains represent truly non-pigment-producing strains of this species. With some atypical strains it may be necessary to test all the eleven properties. The tests for growth on cadmium and tetrazolium media are the simplest and most likely to find routine application.

In conclusion, we recommend the following procedure to identify atypical *Pseudomonas aeruginosa* cultures. First, ascertain that the strain is a Pseudomonas; this is done by examining shape and staining reaction: Gram-negative rod, test for motility by hanging drop: motile, except for mucoid strains, perform the oxidase reaction: immediate positive, test with Hugh & Leifson's medium: oxidative use of glucose and examine for arginase activity: positive. Then proceed with the tests which will prove the identity of *P. aeruginosa*: growth at 42°, at 37°, oxidation of gluconate or production of slime, production of pyocyanin or pyorubin on Sierra's modified medium, growth on tetrazolium and cadmium containing media, production of collagenase, fluorescence under ultraviolet radiation, production of pyocines and slide-agglutination with a polyvalent serum.

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REFERENCES

BLANC. J. (1923). Transformation des bacilles pyocyaniques en bacilles sans pigment. Essai d'interprétation. *C.r. Séanc. Soc. Biol.* **88**, 52.

BURTON, M. O., CAMPBELL, J. J. R. & EAGLES, B. A. (1948). The mineral requirements for pyocyanin production. *Can. J. Res.* (*C*), **26**, 15.

CARPENTER, K. P. (1961). The relationship of the Enterobacterium A 12 (Sachs) to Shigella Boydii 14. *J. gen. Microbiol.* **26**, 535.

CATALIOTTI, F. (1935). Ricerche sul *B. pyocyaneum*. *G. Batt. Immunol.* **15**, 161.

CLARKE, S. K. R. (1953). A simplified plate method for detecting gelatine-liquefying bacteria. *J. clin. Path.* **6**, 246.

DANZ, F. J. & SCHULTZ, E. W. (1949). Gelatinous variants of *Pseudomonas aeruginosa*. *J. Bact.* **58**, 367.

DARRELL, J. H. & WAHBA, A. H. (1964). Pyocine typing of hospital strains of *P. pyocyanea*. *J. clin. Path.* **17**. 236.

FRANK, L. H. & DE MOSS, R. D. (1959). On the biosynthesis of pyocyanin. *J. Bact.* **77**, 776.

FUJITA, H. (1957). Influence of antibiotics on the production of pyocyanin and fluoresein by *P. aeruginosa* and the occurrence of non-pyocyanin strains from clinical specimens. *Mie. Med. J.* **7**, 169.

GABY, W. L. (1955). Taxonomic problems relating to the identification of species within the genus Pseudomonas. *Int. Bull. bact. Nomencl. Taxon.* **5**, 153.

GABY, W. L. & FREE, E. (1953). Occurrence and identification of non-pigmented strains of *P. aeruginosa* in the clinical laboratory. *J. Bact.* **65**, 746.

GESSARD, C. (1919). Classement des germes pyocyaniques par les pigments. *C.r. Séanc. Soc. Biol.* **82**, 795.

GESSARD, C. (1920). Technique d'identification des germes pyocyaniques. *Ann. Inst. Pasteur.* **34**, 88.

HABS, I. (1957). Untersuchungen über die O-Antigene von *P. aeruginosa*. *Z. Hyg. InfektKr.* **144**, 218.

HAMON, Y. (1956). Contribution à l'étude des pyocines. *Ann. Inst. Pasteur*, **91**, 82.

HAYNES, W. C. (1951). *Pseudomonas aeruginosa* characterization and identification. *J. gen. Microbiol.* **5**, 939.

HAYNES, W. C. (1962). Comparative taxonomy of crystallogenic strains of *P. aeruginosa* and *P. chlororaphis*. *J. Bact.* **84**. 1080.

HENRIKSEN, S. D. (1948). Some unusual mucoid organisms. *Acta path. microbiol. scand.* **25**, 485.

HUGH, R. & LEIFSON, E. (1953). The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram-negative bacteria. *J. Bact.* **66**, 24.

KING. E. O., WARD, M. K. & RANEY. D. E. (1954). Two simple media for the demonstration of pyocyanin and fluoresein. *J. lab. clin. Med.* **44**, 301.

KÖHLER, W. (1958). *Pseudomonas aeruginosa*: Cytologie, Biochemie und Serologie. *Wiss. Z. Univ. Rostock*, **7**, 25.

KOVÀCS, N. (1956). Identification of *P. pyocyanea* by the oxidase reaction. *Nature, Lond.* **178**. 703.

LOWBURY, E. J. L., LILLY, H. A. & WILKINS, M. D. (1962). A cabinet for the detection of fluorescent bacterial cultures. *J. clin. Path.* **15**, 339.

LUTZ, A., SCHAEFFER, A. & HOFFERER, M. J. (1958). Caractères biologiques et sensibilité aux antibiotiques de bacilles du genre *Pseudomonas*. *Ann. Inst. Pasteur*, **95**, 49.

MUNOZ, J. & DOUGHERTY, W. J. (1951). Studies on a mucoid strain of *Pseudomonas aeruginosa* and its smooth variant. *Bact. Proc.* p. 73.

OAKLEY, C. L., WARRACK. G. H. & VAN HEYNINGEN, W. E. (1946). The collagenase (κ-toxin) of *Clostridium welchii* type A. *J. Path. Bact.* **48**, 229.

ROGERS, K. B. (1963). *Lancet*, **2**, 682 (Letter).

ROSENFELD, J. & APPEL, G. (1963). The use of neopeptone glycerol agar for the identification of *P. aeruginosa*. *Am. J. med. Techn.* **29**, 189.

SANDVIK, O. (1960). Serological comparison between strains of *P. aeruginosa* from human and animal sources. *Acta path. microbiol. scand.* **48**, 56.

SCHNEIERSON, S. S., AMSTERDAM, D. & PERLMAN, E. (1960). Inhibition of *P. aeruginosa* pigment formation by chloramphenicol and erythromycin. *Antibiotics Chemother.* **10**, 30.

SCHULTZ, E. W. (1947). A gelatinous variant of *P. aeruginosa*. *Proc. Soc. exp. Biol. Med.* **65**, 289.

SCHWARZ, L. H. & LAZARUS, J. A. (1947). An unusual strain of *Pseudomonas aeruginosa*. *J. Bact.* **54**, 30.

SELENKA, F. (1958). Über die Abgrenzung von *P. aeruginosa* gegen *P. fluorescens* und *P. putida* auf TTC Nährböden. *Arch. Hyg.* **142**, 569.

SELIGMANN, E. & WASSERMANN, M. (1947). Induced resistance to streptomycin. *J. Immunol.* **57**, 351.

SHERRIS, J. C., SHOESMITH, J. G., PARKER, M. T. & BRECKON, D (1959). Test for the rapid breakdown of arginine by bacteria: Their use in the identification of pseudomonads. *J. gen. Microbiol.* **21**, 389.

SHKEIR, I. L., LOSSE, F. L. & BAHN, A. N. (1963). The isolation and incidence of *P. aeruginosa* from human saliva. *Bact. Proc.* p. 71.

SIERRA, G. (1957). A simple method for the detection of lipolytic activity of microorganisms and some observations on the influence of the contact between cells and fatty substrates. *Antonie van Leeuwenhoek*, **23**, 15.

SONNENSCHEIN, C. (1927). Die Mucosus-Form des Pyocyaneus Bakteriums, *Bacterium pyocyaneum mucosum*. Ein Beitrag zur experimentellen Bakterienvariation. *Zbl. Bakt.* (*I. Abt. Orig.*) **104**, 365.

TAYLOR, J. J. & WHITBY, J. L. (1964). *Pseudomonas pyocyanea* and the arginine dihydrolase system. *J. clin. Path.* **17**, 122.

TURFITT, G. E. (1926). Bacteriological and biochemical relationships in the pyocyaneus-fluorescens group I. The chromogenic function in relation to classification. *Biochem. J.* **30**, 1323.

WAHBA, A. H. (1963). The production and inactivation of pyocines. *J. Hyg., Camb.* **61**, 431.

WAHBA, A. H. (1964 *a*). Thesis. University of London.

WAHBA, A. H. (1964 *b*). Hospital infection with *Pseudomonas pyocyanea*: an investigation by combined pyocine and serological typing. *B.M.J.* (in the Press).

WAHBA, A. H. (1964 *c*). Metallic sheen in *Pseudomonas aeruginosc*. *Nature, Lond.* **204**, 502.

WAHBA, A. H. & LIDWELL, O. M. (1963). A simple apparatus for colicine typing. *J. appl. Bact.* **26**, 246.

WARNER, P. T. J. C. P. (1950). The iridescent phenomenon of *P. pyocyanea*. *Br. J. exp. Path.* **31**, 242.

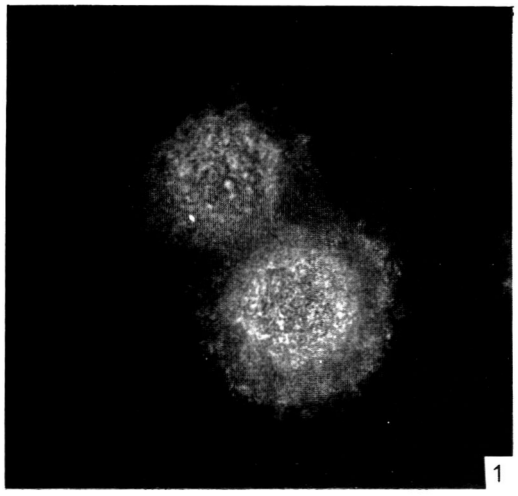
WILLIAMSON, C. K. (1956). Morphological and physiological considerations of colonial variants of *P. aeruginosa*. *J. Bact.* **71**, 617.

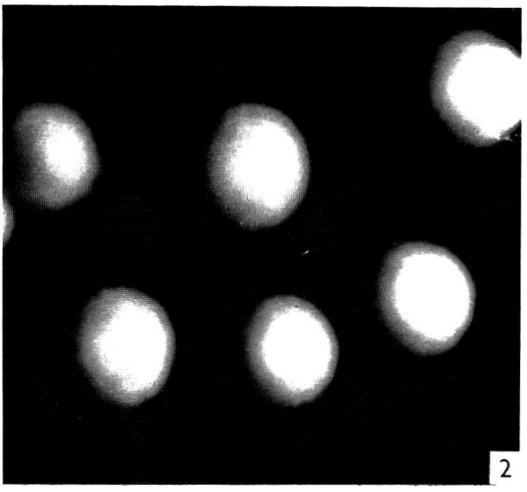
ZIERDT, C. H. & SCHMIDT, P. J. (1964). Dissociation in *Pseudomonas aeruginosa*. *J. Bact.* **87**, 1003.

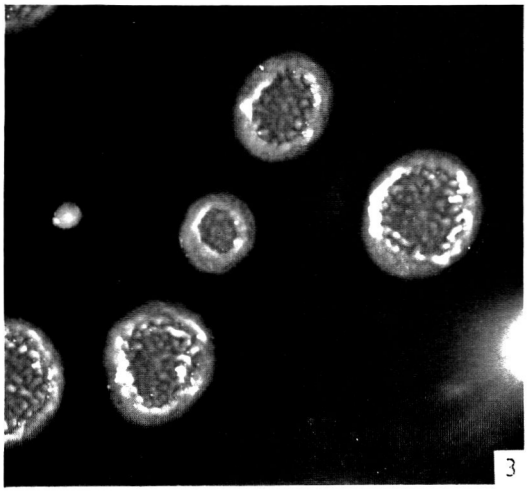
EXPLANATION OF PLATE

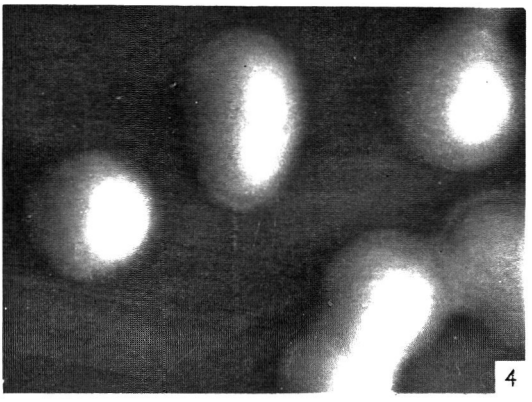
Representative colonial variants of *Pseudomonas aeruginosa* after 24 hr incubation at 37° except for Type 6 which requires 48 hr to produce visible growth. All colonies are from nutrient agar No. 1 cultures.

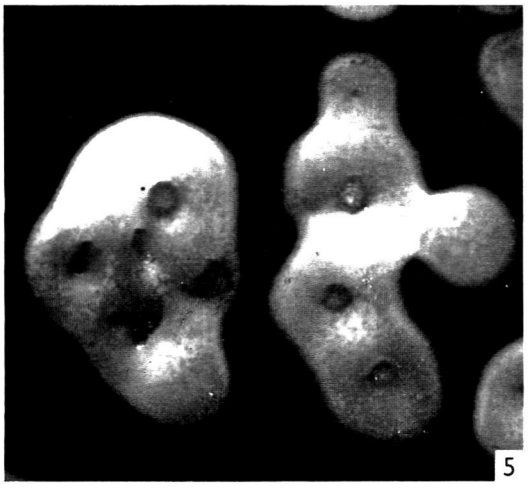
(1) 'S' (smooth) Type X10
(2) 'SR' (smooth-rough) Type X10
(3) 'R' (Rough) Type X10
(4) Mucoid Type X10
(5) Gelatinous Type X10
(6) Dwarf Type X10

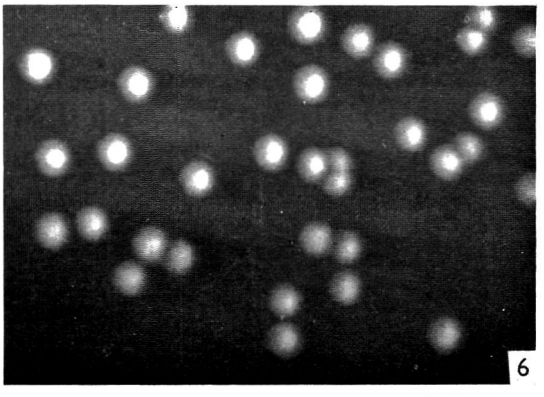












(*Facing p.* 342)

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The Effect Upon *Micrococcus violagabriellae* of Short-Term Exposure to Ultraviolet Light

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SUMMARY

The presence of inorganic or organic iron in the growth medium induces the production of a reddish-violet pigment in *Micrococcus violagabriellae*. This pigment protects the organism against short exposures to ultraviolet (u.v.) radiation of wavelength below 300 mμ. The presence of iron also increases the degree of recovery of the normally white organism after such irradiation. Anaerobic growth renders both the red and the white micrococci more resistant to damage by u.v. radiation. However, the addition of nitrate, an alternative to oxygen as a terminal electron acceptor, restores the u.v. sensitivity of these micrococci to that of aerobically grown organisms. It is suggested that the initial site of u.v. irradiation damage is extranuclear and involves one or more cytochrome-linked systems, and that it is within this system(s) that the iron functions both as a radiation-protective and as a restorative agent.

INTRODUCTION

The significance of bacterial pigments as radiation-protective agents has been commented on by several workers for different types of bacteria, e.g. photosynthetic (Sistrom, Griffiths & Stanier, 1956), non-photosynthetic (Mathews & Sistrom, 1959) and halophilic (Dundas & Larsen, 1962). Studies of the radiation-protection of bacteria attributable to pigment have of necessity been comparisons between pigmented wild-type organisms and non-pigmented mutants. For example, Duggan, Anderson & Elliker (1963) reported that decrease of pigmentation in their strain RW of *Micrococcus radiodurans* was correlated with an increase of sensitivity to γ-radiation. In such comparisons genetic events which govern functions other than pigment production may also be involved. Pigment formation induced by some simple means not involving mutation might be of value in elucidating the mechanism of radiation-sensitivity. Studies along these lines have been made by Cohen–Bazire & Stanier (1958) and Fuller & Anderson (1958) who used diphenylamine to inhibit carotenoid synthesis in the photosynthetic bacterium *Rhodopseudomonas spheroides*. Such inhibition increased the sensitivity of the organisms to the lethal effect of visible light. A reversible system was involved since removal of diphenylamine allowed carotenoid synthesis to resume.

The presence of ferric and/or ferrous ions in growth media induces the production of a reddish-violet pigment in the otherwise white bacterium *Micrococcus violagabriellae* (Campbell, unpublished results, 1957; Cassingena, Ortali & Milazzo, 1960). The ready alteration of pigment production in this organism without the complications

of mutation thus provides a unique system for the study of the effects of the presence or absence of pigment on sensitivity to radiation. Accordingly, we took advantage of this system to compare the effects of u.v. irradiation on red-pigmented and white organisms in the hope that such a comparison might furnish information about the site of irradiation damage.

METHODS

Organism. The organism used was *Micrococcus violagabriellae*, isolated originally by Castellani (1955), and obtained from the culture collection of the American Meat Institute Foundation, Chicago, Illinois, U.S.A.

Media. (*a*) Basal medium (g./l. distilled water): Casamino acids (Difco), 10; yeast extract (Difco), 0·3; cysteine, 0·1; tryptophan, 0·1; asparagine, 0·1; sodium citrate, 5; glucose, 5; KH_2PO_4, 5; NaCl, 5; $MgSO_4.7H_2O$, 0·8; $MnCl_2.4H_2O$, 0·14; agar (Difco), 15; $FeCl_3$ (when present), 0·1.

(*b*) Trypticase soy agar (BBL) + (g./l. medium): glucose, 2·5; $FeCl_3$ (when present), 0·1.

Other substances were added as indicated in the text; the media were adjusted to pH 7·2 before autoclaving (120°, 20 min.) Organisms were grown aerobically, or anaerobically (95% (v/v) N_2 + 5% (v/v) CO_2 atmosphere with reduced methylene blue as indicator of anaerobiosis.)

The incubation temperature was always 30°.

Plating techniques. The problem of clumping, which affects viable counts of micrococci, was overcome as follows; 18 hr organisms were removed from solid media with sterile 0·03M-phosphate buffer (pH 7·2), washed and resuspended in buffer. The suspension was centrifuged for 15 min. at 1000 rev./min. to sediment clumped cocci. Samples were removed from the upper regions of the tubes and added to sterile buffer to yield a turbidity reading of 1·0 on a Spectronic 20 colorimeter (Bausch & Lomb) (equivalent to 4×10^8 cocci/ml.). Phase microscopic examination showed that this suspension consisted nearly exclusively of single cocci. Serial 10-fold dilutions of this suspension were then made to $1/10^7$ and 1·0 ml. samples of the desired dilutions were spread on plates of appropriate media; all platings were made in triplicate. Plates were incubated at 30° for 48 hr at which time the colonies were counted. By this method it was possible to obtain reproducibility within 10%, and this value was taken to be the maximum permissible degree of variation.

Irradiation techniques. (*a*) *Ultraviolet radiation.* A 15 W. germicidal lamp (General Electric) giving a minimum of 90% emission at 260 mμ at a distance of 40 cm. from the surface to be irradiated was the source of ultraviolet (u.v.) radiation. This arrangement yielded a dosage of 102 ergs/sec./cm². at the irradiated surface as measured by a model SM 200 U.V. click meter (Westinghouse Electric Corp., Bloomfield, N.J., U.S.A.). Routinely a 5 sec. exposure was given because it killed about 70% of the cocci of the most sensitive system tested and hence permitted detection of alterations in sensitivity or resistance. Longer exposure times were used, as indicated, to obtain mortality curves, or in those experiments which used filters when the exposure time was increased to 7 sec. to compensate for energy loss in the filter.

Cocci were prepared for irradiation in one of two ways. The buffered suspension of washed centrifuged cocci was placed in a flat bottomed Petri dish to give a layer of suspension not more than 2 mm. thick and the plate gently agitated during irradiation. Dilutions were then made, plated and the colonies counted. In other experiments dilutions of the original suspension were plated and irradiated on the surface of the medium.

In experiments which required the exclusion of far u.v. wavelengths $8 \times 8 \times 0{\cdot}4$ cm. glass filters (Corning Glass Co.) were placed in an opaque frame and mounted 1 cm. from the irradiated agar surface. The characteristics of these filters are summarized in Table 1.

Table 1. *Transmittance characteristics of u.v. filters*

Filter	Transmitting (range mμ)	$\lambda_{max.}$* (mμ)	% of input transmitted at $\lambda_{max.}$
7–51	295–425	365	80
	690–	750	40
7–59	290–490	360	90
	690–	750	40

* $\lambda_{max.}$ wavelength at which maximum transmission occurs.

Cocci were irradiated anaerobically in a gas tight $100 \times 65 \times 50$ cm. glass-fronted inoculating hood fitted with long-sleeved rubber gloves. Plates to be irradiated were placed in the hood some time before irradiation and a continuous flow of N_2 (de-oxygenated by passage through alkaline pyrogallol) was maintained through the hood during irradiation. Reduced methylene blue again served as the test for anaerobiosis.

(*b*) *Visible light.* Studies of the lethal effect of visible light used a 500 W tungsten photoflood lamp (General Electric). Organisms to be illuminated were placed in glass tubes and immersed in a glass-walled constant temperature ice-water bath to prevent heating. Samples were placed 37 cm. from the light source and agitated periodically during illumination. In all cases non-illuminated control tubes wrapped in aluminium foil were held alongside the illuminated samples. Dilutions and plating were carried out as described.

Nitrate reduction. The accumulation of nitrite in culture media was measured by using the sulphanilic acid + α-naphthylamine spot test (Pelczar, 1957); the colour development was estimated visually.

RESULTS

The production of the characteristic reddish-violet pigment of *Micrococcus violagabriellae* is dependent on the presence of oxygen and inorganic iron; the iron is incorporated into the pigment molecule (Payne & Campbell, 1962, 1963). The effect of iron on the ability of the micrococci to withstand u.v.-irradiation damage was measured. The results (Table 2) showed that 74% of the cocci grown in the absence of iron (i.e. white cocci) were killed by 5 sec. exposure to u.v. irradiation, whereas cocci grown in media containing iron (i.e. red cocci), suffered only a 28% mortality under identical conditions. Furthermore, the presence of iron in the post-

irradiation medium decreased the lethality of the u.v. irradiation for both red and white cocci, indicating that iron not only protected the cocci but also aided recovery from irradiation damage.

Optimal protection against u.v.-radiation damage was achieved by using $FeCl_3$ (100 μg./ml.) in the medium. This concentration was also optimal for pigment production and, since higher concentrations of iron neither increased resistance to u.v. irradiation nor increased the amount of pigment, this concentration was used routinely thereafter. No concentration of iron used affected the viability of unirradiated control organisms.

Table 2. *The effect of inorganic iron upon pigment production and resistance to u.v. irradiation*

$FeCl_3$ in inoculum medium (mg./ml.)	Pigmentation of inoculum organisms	$FeCl_3$ added to post-irradiation medium (mg./ml.)	Survivors (%)
0	White	0	26·0
0	White	100	61·3
100	Red	0	71·6
100	Red	100	81·6

Table 3. *The influence of type of iron-containing compound on the resistance of Micrococcus violagabriellae to u.v. radiation for 5 sec.*

Inoculum		Survivors on various post-irradiation media (%)				
				Basal +		
Growth medium	Pigmentation	0	$FeCl_3$*	HB.†	Dial. Hb.‡	Haemin§
Basal +						
0	White	25·5	63·1	82·0	71·0	76·4
$FeCl_3$*	Red	71·4	80·1	87·9	77·0	81·0
Hb.†	White	77·3	89·7	—	—	—
Dial. Hb.‡	White	63·4	84·8	—	—	—
Haemin§	White	76·7	86·2	—	—	—

* $FeCl_3$ = 100 μg./ml.; † HB. = Haemoglobin, 6 mg./ml.; ‡ Dial. Hb. = Dialysed haemoglobin, 6 mg./ml.; § Haemin = 2·5 mg./ml.

To ascertain the dependence upon type of iron, various organic sources of iron were substituted for $FeCl_3$, to yield equivalent final concentrations of Fe. The results (Table 3) showed that although the organic iron sources did not support pigment formation they did confer a degree of protection to u.v. irradiation comparable to that obtained from inorganic iron.

The lethal effect of brief exposure to u.v. radiation might result from damage to some intracellular mechanism or from some irradiation-induced alteration to the extracellular environment which rendered it toxic. Accordingly, experiments were made in which red and white cocci were placed on duplicate sets of media, one half of which had been irradiated for 10 sec. immediately before inoculation. The inoculated plates then received a further 5 sec. irradiation. The results (Table 4) showed that pre-irradiation of the media had no demonstrable effect on survival.

Both red and white cocci were observed to produce catalase under aerobic

conditions of growth, as evidenced by the evolution of gas upon exposure of the cocci to 3% H_2O_2 solution.

It was observed that when white cocci (the most sensitive system) were u.v. irradiated in buffer, and then plated on iron-free medium, only 15·5% survived, compared with 36% if the organisms were plated on iron-containing medium. These results lead to two conclusions: First, iron need not be present at the moment of irradiation in order to effect a modification in sensitivity, but will still function when it is supplied shortly thereafter. For iron to enhance recovery from the u.v.-radiation damage incurred under these conditions, the cocci must be exposed to iron within approximately 5 min. after irradiation. When this time interval was prolonged the ability of the iron to increase survival rates diminished, presumably as the u.v.-induced lesion(s) became irreversible. Thus, the iron in the post-irradiation medium functioned by abetting repair of u.v.-damaged mechanism(s) and not as a passive protective agent. Secondly, the presence or absence of other medium constituents at the time of irradiation did not affect the behaviour of the cocci under these conditions. Hence the technique of u.v.-irradiating cocci on the surface of the growth medium is valid.

Table 4. *The effect of u.v.-irradiation of the medium, before inoculation of Micrococcus violagabriellae, on resistance to subsequent u.v. irradiation*

Inoculum	Post-irradiation media	Pre-inoculation irradiation of medium (sec.)	Survivors (%)
White micrococci	Basal + 0	0	53·0
		10	54·5
	$FeCl_3$*	0	75·0
		10	73·4
	Hb.†	0	82·8
		10	81·0
	Dial. Hb.‡	0	78·0
		10	71·6
	Haemin§	0	72·0
		10	69·1
Red micrococci	0	0	81·4
		10	78·8
	$FeCl_3$	0	80·1
		10	82·6
	Hb.	0	87·9
		10	89·8
	Dial. Hb.	0	77·0
		10	75·4
	Haemin	0	81·0
		10	85·0

* $FeCl_3$ = 0·1 mg./ml.; † Hb. = haemoglobin, 6 mg./ml.; ‡ Dial. Hb. = dialysed haemoglobin, 6 mg./ml.; § Haemin = 2·5 mg./ml.

The report of Kashket & Brodie (1962) on the lethal effects of long-term exposure of *Escherichia coli* and *Pseudomonas aeruginosa* to near-u.v. radiation (360 mμ) made it imperative to test the effects of these longer wavelengths upon the *Micrococcus*

violagabriellae system. This was done in two ways: (i) by using a tungsten light source; (ii) by using the source of u.v. radiation but with appropriate filters.

Repeated attempts to show inactivation of the micrococci by illumination with intense visible light for periods up to 2 hr were unsuccessful The survival rate of cocci in buffer under illumination was in all cases the same as that of the cocci in the dark; and the presence or absence of red pigment and/or exogenous iron had no modifying effect. No photo-reactivation of *Micrococcus violagabriellae* after short term exposure to u.v.-radiation was observed. Table 5 shows that when cocci were u.v. irradiated for 5 sec. and then exposed to intense visible light for 30 min. their

Table 5. *Effect of visible light upon u.v.-irradiated Micrococcus violagabriellae*

Organism	Post-irradiation media	Time of exposure to u.v. radiation (sec.)	Time of exposure to visible light (min.)	Survivors (%)
White	Basal	5	—	37·3
		5	30	34·0
	Basal + Fe	5	—	68·0
		5	30	65·6
Red	Basal	5	—	47·9
		5	30	53·5
	Basal + Fe	5	—	68·5
		5	30	64·8

Table 6. *Effect of elimination of short wavelengths on lethal effect of u.v. radiation on Micrococcus violagabriellae*

Organisms	Post-irradiation media	Irradiation conditions	Survivors (%)
White	Basal	No filter	34·0
		Filter 1	94·5
		Filter 2	101·5
	Basal + Fe	No filter	54·0
		Filter 1	94·7
		Filter 2	98·0
Red	Basal	No filter	64·2
		Filter 1	101·4
		Filter 2	101·0
	Basal + Fe	No filter	84·0
		Filter 1	100·8
		Filter 2	96·5

survival was not significantly different from that of cocci held in the dark under identical conditions after exposure to u.v. radiation. This lack of significant photo-reactivation serves to differentiate the effects of short-term exposure to u.v. radiation seen in these experiments with *M. violagabriellae*, from those observed with other organisms and leads to the consideration of a possible extranuclear site of damage as one of the initial sites of u.v. action.

Table 6 shows the results of an experiment in which the response of micrococci to 5 sec. exposure to unfiltered u.v. radiation was compared to that of micrococci which

received an equivalent dose of u.v. radiation of wavelengths > 300 mμ from the same source (the wavelengths below 300 mμ being eliminated by filters). The presence of pigment and/or exogenous iron again exerted no modifying effect. Consequently the observed lethal effect results from wavelengths below 300 mμ.

Since atmospheric O_2 is required by *Micrococcus violagabriellae* for pigment synthesis, the role that O_2 might play in the sensitivity of this organism to u.v. radiation was examined. Aerobically-grown and anaerobically-grown micrococci were compared with respect to their sensitivity to u.v. irradiation for 5 sec., and the effect of post-irradiation anaerobiosis was studied. The irradiation procedure itself was done in the presence of air. The results (Table 7) show that cocci grown anaerobically in the absence of iron were about 2·5 times more resistant to u.v. radiation than were their aerobically grown counterparts. Addition of iron to the pre-irradiation medium decreased this difference in sensitivity to some 1·5 times, but the increased resistance conferred by anaerobic growth was still evident. The substitution of organic iron sources for inorganic ones did not alter this observation. Anaerobiosis after u.v. irradiation had no effect on survival in any of these systems.

Table 7. *The effect of anaerobiosis on resistance of Micrococcus violagabriellae to u.v. irradiation*

Pre-irradiation incubation	Post-irradiation Media	Post-irradiation incubation	Survivors (%) White pigmented cells	Survivors (%) Red pigmented cells
	Basal +			
Aerobic	0	Aerobic	30·8	68·9
		Anaerobic	32·9	63·1
	$FeCl_3$*	Aerobic	51·5	82·0
		Anaerobic	52·4	83·1
	Hb.†	Aerobic	69·3	88·3
		Anaerobic	72·2	82·6
	Dial. Hb.‡	Aerobic	70·3	85·6
		Anaerobic	80·0	88·0
	Haemin§	Aerobic	60·8	83·5
		Anaerobic	62·0	78·5
Anaerobic	0	Aerobic	69·0	79·8
		Anaerobic	62·0	78·8
	$FeCl_3$	Aerobic	81·5	90·1
		Anaerobic	86·1	87·8
	Hb.	Aerobic	81·1	79·9
		Anaerobic	82·6	80·4
	Dial. Hb.	Aerobic	89·0	88·7
		Anaerobic	91·0	87·3
	Haemin	Aerobic	86·0	80·3
		Anaerobic	78·4	81·9

* $FeCl_3$ = 100 μg./ml.; † Hb. = haemoglobin, 6 mg./ml.; ‡ Dial. Hb. = dialysed haemoglobin, 6 mg./ml.; § Haemin = 2·5 mg./ml.

The next question concerned the importance of O_2 at the time of exposure of the micrococci to u.v. radiation. Experiments were made wherein cocci from various sources were irradiated in an inoculating hood under de-oxygenated N_2 and their sensitivity compared to that of duplicate systems irradiated in air. The results

(Table 8) indicated that the absence of O_2 at the time of irradiation did not alter resistance or ability of the cocci to recover from u.v.-radiation damage.

In summary, the experiments showed that the presence or absence of O_2 was effective only during the period of growth of the micrococci immediately before u.v. irradiation. The presence or absence of O_2 at, or subsequent to the time of irradiation was immaterial to the ability of the micrococci to withstand or to recover from u.v.-radiation damage.

One role of O_2 in the life of these micrococci is as a terminal electron acceptor in aerobic metabolism. The effect of substitution of suitable alternative electron acceptor(s) upon this system was examined. Methylene blue would function in this respect but KNO_3 was found to be superior and it was used routinely. Tubes of basal

Table 8. *Effect of oxygen on u.v. sensitivity of Micrococcus violagabriellae*

Inoculum		Post-irradiation media	Irradiation gas-phase	Survivors (%)
Organism	Growth condition			
White	Aerobic	Basal	Air	42·4
			N_2	45·5
		Basal + Fe	Air	58·2
			N_2	57·8
	Anaerobic	Basal	Air	70·0
			N_2	70·0
		Basal + Fe	Air	70·1
			N_2	69·0
Red	Aerobic	Basal	Air	68·5
			N_2	66·9
		Basal + Fe	Air	77·2
			N_2	81·8
	Anaerobic	Basal	Air	74·2
			N_2	74·7
		Basal + Fe	Air	74·0
			N_2	71·9

Table 9. *Effect of nitrate and/or fluoride on growth and pigment production of Micrococcus violagabriellae*

Growth medium	Incubation conditions	Growth response (absorbance at 600 mμ)	Accumulation of nitrite	Production of red pigment
Basal +				
0	Aerobic	1·3	−	−
	Anaerobic	0·48	−	−
KNO_3	Aerobic	1·2	+ + +	−
	Anaerobic	0·8	+ +	−
NaF	Aerobic	1·3	−	−
	Anaerobic	0·18	−	−
$NaF + KNO_3$	Aerobic	1·4	+ + +	−
	Anaerobic	0·9	+ +	−
$FeCl_3$	Aerobic	1·3	−	+ + + +
	Anaerobic	0·52	−	−
$FeCl_3 + KNO_3$	Aerobic	1·3	+ + +	+ + + +
	Anaerobic	0·75	+ + +	−
$FeCl_3 + NaF$	Aerobic	1·28	−	+ + + +
	Anaerobic	0·21	−	−
$FeCl_3 + KNO_3$ + NaF	Aerobic	1·3	+ + +	+ + + +
	Anaerobic	0·75	+ +	−

medium, with or without $FeCl_3$, KNO_3 (0·01 M), and/or NaF (0·01 M) were prepared, inoculated, and incubated aerobically or anaerobically. Growth response and nitrite accumulation were measured. Plates of corresponding solid media were inoculated under identical conditions to check on pigment production which was estimated visually. The results (Table 9) showed that the presence of 0·01 M-KNO_3 inhibited neither growth nor pigment production of aerobically grown micrococci. That NO_3^- was functional as an electron acceptor was supported by two observations (i) NO_2^- accumulated aerobically and anaerobically (no evidence was obtained indicating ability of the micrococci to further reduce NO_2^-); (ii) the stimulation in aerobic growth response which occurred when NO_3^- was added to the medium. Sodium fluoride (0·01 M) functioned as an inhibitor only under anaerobic conditions and this inhibition was completely reversed by the NO_3^-.

Table 10. *Effect of nitrate on u.v. sensitivity of Micrococcus violagabriellae*

Inoculum			
Growth medium	Growth conditions	Post-irradiation medium	Survivors (%)
Basal +			
0	Aerobic	Basal	41·6
		Basal + Fe	78·7
	Anaerobic	Basal	78·1
		Basal + Fe	75·2
Fe	Aerobic	Basal	74·3
		Basal + Fe	92·2
	Anaerobic	Basal	88·2
		Basal + Fe	83·5
NO_3	Aerobic	Basal	39·1
		Basal + Fe	87·9
	Anaerobic	Basal	45·5
		Basal + Fe	75·1
Fe + NO_3	Aerobic	Basal	72·6
		Basal + Fe	82·0
	Anaerobic	Basal	69·9
		Basal + Fe	77·6

Having established NO_3^- functioned as an alternative electron acceptor, experiments were made to see whether it would further mimic oxygen by restoring u.v. sensitivity to anaerobically-grown micrococci. Table 10 shows the results of such experiments; the effect of iron on sensitivity to u.v. radiation was again evident. Nitrate produced no significant change in the u.v. sensitivity of aerobically-grown micrococci. However, the presence of NO_3^- in the medium of micrococci grown in the absence of O_2 restored the degree of u.v. sensitivity of these micrococci to that of their aerobically-grown counterparts. This confirmed that NO_3^- could substitute for O_2 in all regards as an electron acceptor.

Previous work with this organism was concerned with the effect of various inhibitors upon growth and pigment formation (Campbell, Nichols & Berry, 1964). Table 11 shows the effect on sensitivity to u.v. radiation of adding some of these inhibitors to the growth media. The concentrations used had been found to affect

the pigment production but not the growth of the organisms. The data from micrococci grown in the absence of iron showed that neither glutathione nor *o*-cresol affected sensitivity or recovery. Ascorbic acid decreased the sensitivity somewhat but did not affect recovery. With micrococci grown in presence of iron, ascorbic acid inhibited pigment production only slightly, with the result that resistance to u.v. radiation was high. Glutathione partially inhibited pigment production and by so doing increased the sensitivity of these micrococci to a value intermediate between those of red and white micrococci. *O*-cresol completely inhibited pigment formation and thereby rendered the micrococci most sensitive to u.v. radiation. The correlation between the degree of inhibition of pigment formation and the degree of increase of sensitivity to u.v. radiation is apparent.

Table 11. *Effect of various inhibitors on pigment production and u.v. sensitivity of Micrococcus violagabriellae*

Concentration of additions: glutathione, 0·4 mg./ml.; ascorbic acid, 0·8 mg./ml.; *o*-cresol, 1·8 mg./ml.; Fe^{2+} (as $FeCl_3$), 0·1 mg./ml.

Inoculum			
Growth medium	Pigment formed	Post-irradiation medium	Survivors (%)
Basal +			
0	White	Basal	48·6
		Basal + Fe	64·5
Glutathione	White	Basal	49·5
		Basal + Fe	70·2
Ascorbic acid	White	Basal	59·3
		Basal + Fe	80·2
o-cresol	White	Basal	52·5
		Basal + Fe	63·0
Fe	Red	Basal	75·9
		Basal + Fe	91·8
Fe + glutathione	Red	Basal	70·0
		Basal + Fe	81·8
Fe + ascorbic acid	Red	Basal	95·6
		Basal + Fe	100·0
Fe + *o*-cresol	White	Basal	45·5
		Basal + Fe	70·0

Preliminary experiments indicated that the modifying influences on sensitivity to u.v. radiation of this organism tended to become less pronounced when the dosage of u.v. radiation was increased. The response of the micrococci to different doses of u.v. radiation (given by different exposure times) was examined. Examples of typical survival curves are given in Fig. 1. These experiments produced curves which compared the effect of: (*a*) presence or absence of red pigment: (*b*) presence or absence of iron in the post-irradiation medium; (*c*) anaerobic or aerobic growth of inoculum; (*d*) presence or absence of NO_3^- in the pre-irradiation medium of anaerobically-grown organisms. In all cases the two-phase curve shown in Fig. 1 was obtained. Furthermore, the differences in behaviour occurred in the response to u.v. irradiation for 5–10 sec. After this time the survival curves became parallel in all cases. This is seen by comparing the slopes of the curves in Fig. 1 from 10 sec. onward; curve 1, 1·44: curve 2, 1·42: curve 3, 1·20: curve 4, 1·26.

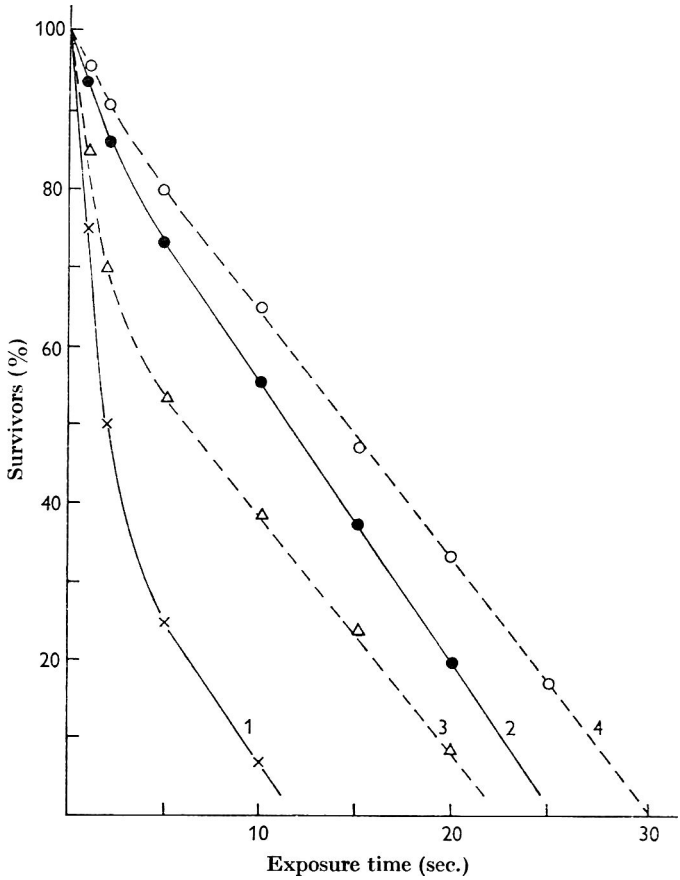

Fig. 1. Effect of increasing time of exposure to u.v. radiation on viability of Micrococcus violagabriellae. Curve 1(× —— ×), white micrococci on iron-free post-irradiation medium; Curve 2 (● —— ●), red micrococci on iron-free post-irradiation medium; Curve 3 (△— —△), white micrococci on iron-containing post-irradiation medium; Curve 4 (○— —○), red micrococci on iron-containing post-irradiation medium.

DISCUSSION

The data showing the protective effect of the red pigment, and the modification of this effect with inhibitors, connect the red pigment, or its formation, with the site of u.v.-radiation damage in this organism. That the lethal effect of short-term u.v. radiation involves an iron dependent system is shown by the fact that protection is conferred not only by the presence of iron containing pigment which has been identified as pulcherrimin (Campbell *et al.* 1964) but also by sources of iron that do not support pigment production.

The results from the use of the inhibitors permit speculation about this system. For micrococci growing in the absence of iron, hence not synthesizing red pigment, glutathione had no effect on sensitivity to u.v. radiation which suggests that a sulphydryl system was not involved. *O*-cresol similarly had no effect on non-pigmented micrococci. Both of these substances can affect sensitivity to u.v.

radiation only under conditions where their presence depresses the amount of pigment synthesized, and hence the amount of iron complexed by the organisms. Ascorbic acid decreased the sensitivity of the micrococci to u.v. radiation and, since it is a reducing agent, perhaps it protected an oxidation-reduction system.

Oxygen was shown to play a key role in sensitivity to u.v. radiation. There are two possible roles it might perform. (*a*) As a component of peroxide production. The production of peroxide(s) as an explanation for the lethal effect of u.v. radiation is ruled out for several reasons. No difference in catalase production between red and white micrococci was detected which might account for the different sensitivities. This agrees with the observations of Adler (1963) who found no correlation between resistance to ionizing radiation and catalase content in *Escherichia coli* mutants. The presence or absence of O_2 at the moment of u.v. irradiation, which would be critical for u.v.-dependent peroxide production, had no effect upon the u.v. sensitivity of *Micrococcus violagabriellae*; and u.v. irradiation of the medium, even for twice the standard interval of time, did not make it toxic.

(*b*) The second possible role of O_2 might be as a terminal acceptor in the cytochrome-linked electron transfer chain. This seems the most likely role of O_2 since the substitution of another usable electron acceptor such as NC_3^- for O_2, completely restored the u.v. sensitivity of anaerobically-grown *Micrococcus violagabriellae*. This supposes that *M. violagabriellae* uses one metabolic pathway while growing anaerobically and a different pathway, sensitive to brief exposure to u.v. radiation, when growing in the presence of O_2 or NO_3^-. The results of the experiments with NaF support this. Fluoride is a selective inhibitor of the glycolytic pathway, inhibiting enolase activity (Warburg & Christian, 1942). Since fluoride did not inhibit either aerobic growth or pigment formation by *M. violagabriellae* it is assumed that aerobic mechanisms were unaffected by the concentration used, and that glycolysis represented an insignificant fraction of the activity of the micrococci under aerobiosis. However, under anaerobiosis the micrococci must rely more heavily on the glycolytic mechanism and fluoride then becomes a potent inhibitor. The fact that both O_2 and NO_3^- functioned in the stimulation of growth, the annullment of fluoride inhibition, and the increase of u.v. sensitivity of the organisms, is evidence for the similarity of their roles.

It would appear then that the sensitivity of the organisms to short term exposure to u.v. radiation is associated with the presence in the micrococci, at the time of irradiation, of an iron dependent oxidation-reduction system which will use either O_2 or NO_3^- as a terminal electron acceptor. This might be a shared cytochrome-linked electron transfer chain.

If *Micrococcus violagabriellae* is grown aerobically, or anaerobically in the presence of NO_3^-, the organisms will preferentially use the 'aerobic', that is, the more efficient cytochrome-linked system, and as a result be metabolically committed to a greater extent to this system than are their anaerobically grown counterparts. If the site of u.v.-radiation damage is somewhere along this path and if this mechanism is destroyed, then the aerobically grown micrococci have had a greater percentage of the metabolic machinery damaged, i.e. have suffered a larger lesion(s) with respect to total respiratory activity, and hence have a lower probability of recovery than anaerobically-grown micrococci which have not relied on that mechanism for a

significant part of their activity. The protective and restorative effects of iron in this regard are obvious.

It has been observed that O_2 and respiratory NO_3^- reduction share cytochrome-linked pathways of electron transport in several micro-organisms (Taniguchi, Sato, & Egami, 1956; Sadana & McElroy, 1957) and that O_2 tension of the medium can dictate the alternate route of terminal electron transfer used (Lenhoff, Nicholas & Kaplan, 1956). Berliner (1963) showed that u.v. radiation produced reversible changes in luminescence (an electron transfer system) in the basidiomycete *Armillaria mellea*. This permits the hypothesis that sensitivity to u.v. radiation in *Micrococcus violagabriellae* is correlated with differences in electron transfer systems resulting from different growth conditions.

The effects of u.v. radiation on cell nuclear material have been extensively studied and established. It is not claimed that this damage does not occur in *Micrococcus violagabriellae* but rather that there is also a mechanism(s) involving electron transport which is susceptible to lower degrees of u.v. irradiation than those associated with damage to the deoxyribonucleic acid fraction. This agrees with the findings of Canzanelli, Sossen & Rapport (1957) who showed that u.v. irradiation of rat liver mitochondria depressed succinoxidase and cytochrome oxidase activity, and that the energy necessary to effect such changes was much less than that necessary to produce chemical changes in nucleic acid derivatives. In *M. violagabriellae* it is solely with the 'cytochrome' site that the modifying effects exhibited by anaerobiosis, iron, etc., are associated. Examination of the survival curves shows that in all cases, the differences between red and white micrococci, aerobically- and anaerobically-grown micrococci, u.v.-irradiated micrococci placed in iron-containing and iron-deficient media, etc., are reflected in the response of the organisms to the first 10 sec. or so of u.v. irradiation. As the doses increased, in all cases the curves became essentially parallel, the differences being a reflexion of tolerance of the low dosage. At the higher dosage the nuclear sites, which are not affected by iron, O_2 tension, etc., are irreversibly damaged and the modifying effect of the above mentioned parameters ceases.

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REFERENCES

Adler, H. I. (1963). Catalase, hydrogen peroxide, and ionizing radiation. *Rad. Res.*, Suppl. **3**, 110.

Berliner, M. D. (1963). The action of monochromatic ultraviolet radiation on luminescence in *Armillaria mellea*. *Rad. Res.* **19**, 392.

Campbell, J. N., Nichols, J. L. & Berry, S. A. (1964). Characterization of the pigment of *Micrococcus violagabriellae*. *Can. J. Microbiol.* **10**, 659.

Canzanelli, A., Sossen, R. & Rapport, D. (1957). Succinic and cytochrome oxidase activity of rat liver mitochondria after *in vitro* irradiation with ultraviolet light. *Am. J. Physiol.* **188**, 547.

Cassingena, R., Ortali, A. V., Villa, L. & Milazzo, M. (1960). *Micrococcus violagabriellae* (Castellani, 1955). *Rend. Ist. Super. di Sanità*, **23**, 1.

CASTELLANI, A. (1955). Note préliminaire sur un noveau micrococque isolé d'une dermatite axillaire superficielle tropicale. *Ann. Inst. Pasteur*, **89**, 475.

COHEN-BAZIRE, G. & STANIER, R. Y. (1958). Specific inhibition of carotenoid synthesis in a photosynthetic bacterium and its physiological consequences. *Nature, Lond.* **181**, 250.

DUGGAN, D. E., ANDERSON, A. W. & ELLIKER, P. R. (1963). Inactivation of radiation-resistant spoilage bacterium *Micrococcus radiodurans*. I. Radiation inactivation rates in three meat substrates and in buffer. *Appl. Microbiol.* **11**, 398

DUNDAS, I. D. & LARSEN, H. (1962). The physiological role of the carotenoid pigments of *Halobacterium salinarium*. *Arch. Mikrobiol.* **44**, 233.

FULLER, R. C. & ANDERSON, I. C. (1958). Suppression of the carotenoid synthesis and its effect on the activity of photosynthetic bacterial chromatophores. *Nature, Lond.* **181**, 252.

KASHKET, E. R. & BRODIE, A. F. (1962). Effects of near-ultraviolet irradiation on growth and oxidative metabolism of bacteria. *J. Bact.* **83**, 1094.

LENHOFF, H. M., NICHOLAS, D. J. D. & KAPLAN, N. O. (1956). Effects of oxygen, iron and molybdenum on routes of electron transfer in *Pseudomonas fluorescens*. *J. biol. Chem.* **220**, 983.

MATHEWS, M. M. & SISTROM, W. R. (1959). Function of carotenoid pigments in non-pathogenic bacteria. *Nature, Lond.* **184**, 1892.

PAYNE, J. I. & CAMPBELL, J. N. (1962). The influence of iron on pigmentation and resistance to ultraviolet irradiation in *Micrococcus violagabriellae*. *Bact. Proc.* p. 59.

PAYNE, J. I. & CAMPBELL, J. N. (1963). Concerning the site of ultraviolet sensitivity in *Micrococcus violagabriellae*. *Bact. Proc.* p. 50.

PELCZAR, JR., M. J. (Chairman) (1957). *Manual of Microbiological Methods*, pp. 153–154. New York: McGraw-Hill Co. Inc.

POLLOCK, M. R. (1946). Adaptation of 'nitratase' in washed suspensions of bacteria. *Br. J. exp. Path.* **27**, 419.

SADANA, J. C. & MCELROY, W. D. (1957). Nitrate reductase from *Achromobacter fischeri* purification and properties: Function of flavines and cytochrome. *Arch. Biochem. Biophys.* **67**, 16.

SISTROM, W. R., GRIFFITHS, M. & STANIER, R. Y. (1946). The biology of photosynthetic bacterium which lacks colored carotenoids. *J. cell. comp. Physiol.* **48**, 473.

TANIGUCHI, S., SATO, R. & EGAMI, F. (1956). The enzymatic mechanisms of nitrate and nitrite metabolism in bacteria. In *Symposium in Inorganic Nitrogen Metabolism*. Ed. by W. D. McElroy & B. Glass, pp. 87–108. Baltimore: The Johns Hopkins Press.

WARBURG, O. & CHRISTIAN, W. (1943). Isolierung und Kristallization des Gärungsferments Zymohexase. *Biochem. Z.* **314**, 149.

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Properties of Soluble Antigen of *Trypanosoma evansi*

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SUMMARY

Plasma of rats infected with *Trypanosoma evansi* contained soluble antigen detected by the agar diffusion method. The antigen protected mice against homologous challenge but was not agglutinogenic. Whether the two properties were due to one or two antigens, respectively, was not ascertained. Trypanosome homogenate contained three antigens, two of which were exclusive to the trypanosome, whereas the third antigen was the same as the soluble antigen of the infected rat plasma as established by the gel diffusion and indirect haemagglutination tests.

INTRODUCTION

Weitz (1960*a*, *b*) demonstrated a soluble antigen (exo-antigen) in the serum of rats infected with *Trypanosoma brucei*; it was detectable by gel diffusion technique, protected immunized mice against homologous challenge and was agglutinogenic. Gray (1960) showed soluble trypanosomal antigens in sera of rats infected with *T. vivax*, *T. gambiense* and *T. brucei* which precipitated with anti-*T. vivax* sera of cattle. Seed (1963) isolated two antigens from serum of rats infected with *T. rhodesiense*, one a precipitin which lacked immunizing property, and the other which protected the immunized mice but was not detectable by the agar diffusion method. During work on immunity with *T. evansi*, the author observed similar properties in plasma of rats infected with this protozoon, which are reported in this paper.

METHODS

Strain of the parasite. The Madras strain of *Trypanosoma evansi* was used which has been described previously (Gill, 1964).

Animals. White mice weighing about 15 g. were used for experiments on immunity. Albino rats of 150–200 g. body weight were used for harvesting trypanosomes for antigen.

Infected rat plasma (IRP). Rats at the height of parasitaemia were anaesthetized with chloroform, and bled from the heart with heparin as anticoagulant. Blood from about ten rats was pooled and centrifuged at 850 *g* for 10 min. in centrifuge tubes 18 cm. long and 9 mm. diameter at mouth. The supernatant layer of plasma was pipetted off and immediately Seitz-filtered and stored at $-20°$.

Trypanosome homogenate (*TH*). The top thick white layer of trypanosomes left above red cells after removing the infected plasma (IRP) was removed with pipette

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and transferred to a 50 ml. tube half-filled with cold buffered glucose saline (pH 7·2). The lower pinkish layer of trypanosomes and red cells was similarly removed. The sedimented red cells were resuspended in the glucose saline to original volume and centrifuged as before. The supernatant fluid and the top trypanosome layer were pipetted off and added to the corresponding previous collection. The next pinkish layer was removed and diluted in the glucose saline, added to the similar previous collection and the whole spun as before. The top layer of trypanosomes was collected. The pooled suspension of trypanosomes was counted by the standard haemocytometer technique, and washed thrice in cold distilled water. Trypanosomes were disrupted by two treatments of 15 sec. each with a Mullard type MT 20, MSE ultrasonic disintegrator (45/60 cyc./sec., single phase). This constituted the disintegrated trypanosome homogenate which was lyophilized and stored at −20°. For serological work, 2% disintegrated trypanosome homogenate in cold normal saline was prepared, as described by Seed (1963).

Anti-IRP serum. Anti-IRP sera were prepared in rats and rabbits, following Weitz (1960*b*). Half of the serum samples were frozen immediately at −20°; the rest was lyophilized and stored at −20° and reconstituted with distilled water to the original volume when required.

Anti-trypanosome homogenate sera (anti-TH sera). Rabbits were immunized following the technique described by Seed (1963): two animals each with whole trypanosomes and with trypanosome homogenate, respectively. Rats were immunized by Weitz's (1960*b*) method. Sera were absorbed with normal rat plasma proteins as described by Seed (1963).

Gel diffusion test. The Ouchterlony double diffusion method (1949, 1953) was used with 1·2% agar in normal saline containing 1/1000 thiomersalate. The wells were lined with a thin film of agar (Mansi, 1957) and were about 2 mm. deep. The plates were kept in a moist chamber at room temperature (about 17–20°).

Agglutination test. The technique, described by Cunningham & Vickerman (1962), using a glass plate was used.

Haemagglutination test. The indirect haemagglutination test described elsewhere (Gill, 1964) was used to detect antibody in anti-IRP serum of rats. The test antigen used was sonically prepared trypanosome homogenate clarified by centrifugation for 15 min. at 2520 *g*.

Protection test. To obtain an enhanced immune response, saponin SPL (Johnson, Neal & Gall, 1963) was used with IRP. Two doses of 0·2 ml. plasma mixed with 0·1 ml. saponin solution (1 mg./ml.), a fortnight apart, were given under the neck fold of skin of the mouse. They were challenged intraperitoneally with 1×10^4 trypanosomes/mouse, contained in 0·1 ml. of heparinized infected mouse blood diluted with normal saline. The challenge dose was given 2 weeks after the last immunizing dose. Tail blood was then daily examined for trypanosomes. Each group had ten mice when challenged. It was observed that the protective effect of the inoculum was reflected in the prepatent and survival periods of each group. Once the infection became patent it ran its course unaffected, resulting in about the same patent period in all groups. Therefore, in the section on Results, patent periods have been omitted. Only the prepatent period of the infection and the longevity after challenge of each group is described.

RESULTS

Gel diffusion tests. In agar gel two precipitation bands, one prominent and the other faint, appeared between IRP and anti-IRP serum of rats and rabbits. The anti-IRP sera, however, did not precipitate with normal rat plasma. These observations indicated the presence of a soluble antigen in the IRP. To prove the specificity of this antigen and to elucidate its relationship with antigens of the whole trypanosome, anti-IRP sera and anti-TH serum were side by side titrated against IRP. Results of this experiment are shown in Fig. 1, which has been constructed from the agar plate photographed at intervals to show all the precipitation bands appearing at different speeds.

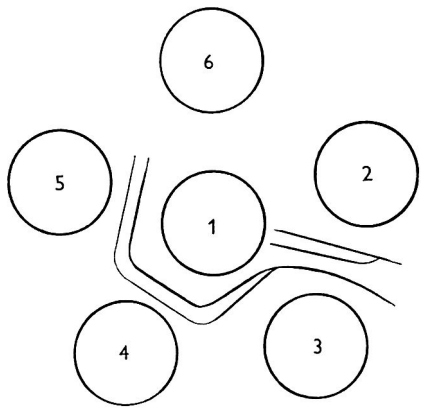

Fig. 1. Double gel diffusion precipitin reactions of the soluble antigen and homogenate of *Trypanosoma evansi* with their antisera. 1. Plasma of rats infected with *T. evansi*. 2. Trypanosome homogenate (*T. evansi*). 3. Anti-trypanosome homogenate (*T. evansi*) serum prepared from rabbit and absorbed with lyophilized normal rat plasma. 4. Anti-IRP serum prepared from rats. 5. Anti-IRP serum prepared from rabbit and absorbed with lyophilized normal rat plasma. 6. Anti-IRP serum of rats absorbed with lyophilized trypanosomes (*T. evansi*).

Figure 1 shows that three bands were formed by the reaction of trypanosome homogenate and anti-TH serum of rabbit absorbed with normal rat plasma proteins, indicating the presence of at least three antigens in the trypanosome. Two bands were precipitated between IRP and anti-TH serum of rabbit, which were identical with the two bands between the IRP and anti-IRP sera. This reveals the presence of the antibody to the soluble antigen both in anti-IRP and anti-TH sera. The two bands due to the soluble antigen at the other end joined one of the three bands of the TH–anti-TH reaction, thus establishing the identity of the soluble antigen with one of the three antigens in the trypanosome homogenate. It appears that this band between TH and anti-TH serum represented two antigen–antibody factors which were resolved into two identical bands between IRP and anti-IRP serum owing perhaps to better conditions present between the latter reactant wells. That the bands represented true trypanosomal antigen–antibody reactions is conclusively proved since (1) the antisera did not contain antibodies to the normal rat plasma as shown by the lack of precipitation line against the normal rat proteins; (2) the absorption of antisera with lyophilized trypanosomes neutralized the anti-

body completely as absorbed sera failed to precipitate with both IRP and trypanosome homogenate. These observations established the identity of the soluble antigen in the plasma of rats infected with *Trypanosoma evansi* with one of the three antigens present in the whole trypanosome.

Agglutination test. Anti-TH sera of two rabbits gave an agglutinating titre of 1/80. The anti-IRP sera, however, did not agglutinate trypanosomes.

Table 1. *Prepatent period and survival time of mice immunized with normal rat plasma and infected rat plasma with or without saponin SPL as adjuvant*

Mice were challenged with 1×10^4 trypanosomes a fortnight after last immunizing dose. Each group consisted of ten mice when challenged with trypanosomes.

Group	Immunizing inoculum	Average prepatent period (days)	Average survival time after challenge (days)
1	No treatment	1·5	5·0
2	Saponin SPL alone	1·4	5·0
3	Infected rat plasma (IRP) alone	5·4	8·9
4	IRP + saponin SPL	7·2	10·8
5	Normal rat plasma (NRP)	1·4	4·9
6	NRP + saponin SPL	1·4	5·1

Haemagglutination test. The anti-IRP serum from rats gave a titre of 1/9000 against the entire soluble antigen complex contained in the trypanosome homogenate. This observation lends further support to the conclusion derived from gel diffusion test that the soluble antigen of the plasma of rats infected with *Trypanosoma evansi* formed a part of the antigenic complex of the trypanosome.

Protection test. Results of the investigation on the protective nature of immunity conferred by the soluble antigen of the IRP are summarized in Table 1. The data given in Table 1 show that the prepatent period of the infection in mice immunized with IRP was significantly longer than that of the unprotected control mice (cf. the prepatent period of 5·4, and 1·4 to 1·5 days, respectively). Therefore, it was concluded that the soluble antigen in the plasma of rats infected with *Trypanosoma evansi* excited the development of immunity which was of a protective character. The immunogenicity of the IRP was considerably enhanced when injected with the adjuvant, saponin SPL, as the prepatent period of the infection in mice immunized thus was further increased to 7·2 days.

DISCUSSION

The whole operation of collection of infected blood from rats and separation of plasma from trypanosomes and its filtration was achieved as rapidly as possible (20 min.) to minimize the possibility of 'contamination' of the plasma with products of disintegrating trypanosomes which might have died after leaving the host. During this time, appreciable number of trypanosomes could not have died since microscopic examination of plasma before filtration showed only active forms. It is therefore reasonable to assume that whatever antigenic trypanosomal factor or

factors were detected in the infected plasma were already there in the blood before it was taken from the rats. It appears that the soluble antigen of the infected plasma might have been secreted or excreted by the living trypanosomes, since this antigen also formed a part of the whole trypanosome antigen as detected by the gel diffusion and haemagglutination tests. The soluble antigen of *Trypanosoma evansi* was precipitinogenic, and partially protected mice against infection with the trypanosome. Whether a single soluble antigen in plasma of rats infected with *T. evansi* excited the development of precipitin and protective antibody, or whether two distinct antigens were involved, as in *T. rhodesiense* (Seed, 1963), was not determined. The antigen was not agglutinogenic.

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REFERENCES

Cunningham, M. P. & Vickerman, K. (1962). Antigenic analysis in the *Trypanosoma brucei* group, using the agglutination reaction. *Trans. Roy. Soc. trop. Med. Hyg.* **56**, 48.

Gill, B. S. (1964). Indirect haemagglutination technique in the study of experimental *Trypanosoma evansi* infections. *Ann. trop. Med. Parasit.* **58**, (in the Press).

Gray, A. R. (1960). Soluble antigens of *Trypanosoma vivax* and of other trypanosomes. *Immunology*, **4**, 253.

Johnson, P., Neal, R. A. & Gall, D. (1963). Protective effect of killed trypanosome vaccines with incorporated adjuvants. *Nature, Lond.* **200**, 83.

Mansi, W. (1957). The study of some viruses by the plate gel diffusion precipitin test. *J. comp. Path.* **67**, 297.

Ouchterlony, O. (1949). Antigen-antibody reactions in gels. *Ark. Kemi Miner. Geol.* **26B**, 1. (Article 14.)

Ouchterlony, O. (1953). Antigen-antibody reactions in gels. IV. Types of reactions in coordinated systems of diffusion. *Acta path. microbiol. scand.* **32**, 231.

Seed, R. (1963). The characterisation of antigens isolated from *Trypanosoma rhodesiense*. *J. Protozool.* **10**, 380.

Weitz, B. (1960*a*). A soluble protective antigen of *Trypanosoma brucei*. *Nature, Lond.* **185**, 788.

Weitz, B. (1960*b*). The properties of some antigens of *Trypanosoma brucei*. *J. gen. Microbiol.* **23**, 589.

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The Classification of Staphylococci and Micrococci from World-wide Sources

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SUMMARY

A study has been made of 607 cultures of Gram-positive and catalase-positive cocci received from workers and collections in different parts of the world. These cultures were examined for a wide range of morphological and physiological characters and representative cultures were further studied to determine the chemical constituents of the organisms. Five hundred and sixty-four of the cultures received were aerobic members of the Micrococcaceae, and of these 96% were classified in the author's groups and subgroups; a further subgroup was, however, introduced to accommodate the not previously studied pink-pigmented micrococci. It appears that the Gram-positive and catalase-positive cocci are best separated into the genus *Staphylococcus* and the genus *Micrococcus* on the ability of members of *Staphylococcus* to grow and produce acid from glucose anaerobically; six subgroups of staphylococci were recognized and eight of micrococci. The relationship of named species, groups and subgroups to the author's classification was examined; several species and groups of micrococci had been incorrectly classified. Thus, *M. denitrificans*, *M. halodenitrificans* and *M. radiodurans* possess characters which suggest that they should be reclassified with the Gram-negative genera and that Abd-El-Malek and Gibson's group IIIB should be classified with the Gram-positive microbacteria.

INTRODUCTION

This paper records the study of 607 cultures of non-halophilic Gram-positive and catalase-positive cocci received from workers and collections in different parts of the world. They were examined by using a wide range of physiological tests; representative organisms were further studied to determine their chemical composition. The organisms studied were named species, groups or subgroups that have been maintained in culture collections or were recent isolates which had been sent to the author as Gram-positive and catalase-positive cocci, or as belonging to the genera *Micrococcus*, *Staphylococcus* or *Sarcina*. Included amongst these organisms were the original strains used in the recent classifications of the Gram-positive and catalase-positive cocci by Abd-el-Malek & Gibson (1948), Shaw, Stitt & Cowan (1951), Pohja (1960), Anderson (1962), Mossel (1962), and Kocur & Martinec (1962). The objects of this work were: to apply the author's proposed classification of the Gram-positive and catalase-positive cocci (Baird-Parker, 1962, 1963) to strains isolated from a wide range of sources; to compare the characters of named species from different collections and habitats; to establish the relationship between the different taxa proposed in recent classifications of these organisms.

METHODS

Table 1 gives the sources of the cultures examined. They had been received on agar slopes or freeze-dried in ampoules. They were checked for purity by plating on the yeast glucose agar described by Baird-Parker (1963) and maintained on slopes of heart-infusion agar stored at 5°; 467 of these cultures were subsequently freeze-dried for further study.

Table 1. *Source of cultures*

Donor	Source	No. of cultures	Description of cultures
Dr J. I. W. Anderson	North sea	8	Marine micrococci
Professor R. Buttiaux	food	4	Staphylococci, micrococci
Miss E. Coster	miscellaneous	7	Named species of aerococci, pediococci
Dr C. M. Cousins	milking equipment	6	Gram-positive catalase-positive cocci
Dr S. T. Cowan	miscellaneous	85	Named species of micrococci, sarcinas, staphylococci, aerococci, pediococci (NCTC collection*)
Dr G. H. Davis	dental	6	Staphylococci
Dr R. H. Deibel	miscellaneous	3	*Micrococcus hyicus*; 'denitrifying' staphylococci
Dr E. I. Garvie	miscellaneous	40	Named species of micrococci, sarcinas, staphylococci (NCDO collection†)
Professor N. G. Heatley	sewage	1	Su's (1948) micrococcin-producing micrococcus
Dr A. J. Holding	acid soils	12	Micrococci, sarcinas
Miss M. John	(1) nose	21	Coagulase-negative staphylococci
	(2) air	50	Gram-positive catalase-positive cocci
Dr M. Kocur	miscellaneous	90	Named species of staphylococci, micrococci
Dr J. Liston	sea foods and marine mud	20	Staphylococci, micrococci
Dr E. J. Lowbury	skin burns	50	Staphylococci, micrococci
Dr I. J. McDonald	Cheddar cheese	10	Cocci
Mr J. H. H. Mol	dry sausage	6	Micrococci
Dr D. A. A. Mossel	food	2	*Staphylococcus aureus*, and a micrococcus
Professor R. G. E. Murray	human	6	'Inhibitory micrococci'
Dr F. P. Ninivaara	dry sausage	1	Micrococcus M53
Mr J. Patterson	pork, Wiltshire bacon and brine	139	Gram-positive catalase-positive cocci
Dr D. M. Pilsbury	human skin	4	*Staphylococcus albus*
Dr M. S. Pohja	(1) fermented meat products	11	Representatives of Pohja's (1960) subgroups
	(2) dry sausage	4	Micrococci
Dr J. W. Rouatt	miscellaneous	6	Named *Micrococcus* spp.
Dr H. Williams Smith	chicken, pig, cow, sheep	15	*S. aureus*
Dr J. M. Shewan	marine	10	Micrococci, cocci (NCMB collection‡)
Dr M. J. Thornley	food	1	*M. radiodurans*
Dr K. Warnøe	bacon	3	*S. aureus*, *S. epidermidis*

* National Collection of Type Cultures; † National Collection of Dairy Organisms; ‡ National Collection of Marine Bacteria.

Examination of morphology and physiological characters

Morphology was examined in hanging-drop preparations and in smears stained by Gram's method. Organisms for these studies were grown at 30° and 37° on yeast glucose agar and nutrient agar, and in nutrient broth. Motility was determined on cultures grown in yeast glucose broth for 6–7 hr at 30°; organisms were subcultured at least once in yeast glucose broth before testing. For studies of physiological characters, organisms were grown at 30° on yeast glucose agar and then subcultured on nutrient agar. Growth from plates of the latter was used to inoculate bottles of nutrient broth. Tests were done by using the media and methods described by Baird-Parker (1963).

Examination of cell and cell-wall components

Forty-five strains of Gram-positive, catalase-positive cocci were examined for sugars, amino sugars, amino acids, teichoic acids, peptides and proteins. Bacteria for these studies were grown by inoculating yeast glucose broth (200 ml.) in 1 l. conical flasks shaken at 120 oscillations/min. for 18–48 hr at 30°. One l. of culture of each organism was grown, giving a yield of 2 to 4 g. wet weight organism. The organisms were washed 3 times with distilled water and then divided into two equal parts. One part was broken to obtain cell-wall and cell-content fractions and the other was used for extracting constituents from whole organisms.

Organisms were broken by shaking with No. 12 ballotini beads at 5° in a Mickle disintegrator; conditions used were those found optimal by Salton & Horne (1951). The ballotini beads were removed by filtration through sintered glass filters (No. 1 porosity) and the cell-wall fragments collected by centrifuging the filtrates at 35,000*g* for 10 min. The supernatant fluids, containing the cell contents of the disrupted organisms, were concentrated to $\frac{1}{3}$ volume by dialysis in Visking dialysis tubing against polyethylene glycol (mol. wt. 20,000) at 5°; they were stored at −20°.

Amino acids. Cell-wall fragments were cleaned by digestion for 18 hr at 37° in 5 ml. of a 0·5 % (w/v) solution of crystalline trypsin (pH 8·6). These were collected by centrifugation at 35,000*g* for 20 min. and after washing 3 times with distilled water they were hydrolysed with 6 N-HCl and further treated by the procedures described by Davis & Baird-Parker (1959). The amino acids were separated by two-dimensional chromatography on Whatman No. 1 filter paper by using the solvent system of Davis & Freer (1960). The papers were developed by spraying with a solution of ninhydrin (0·25 %) in acetone + water + collidine (95 + 5 + 1 by vol.) and heating at 105° for 5–10 min. Isomers of diaminopimelic acid were separated by using the solvent system of Hoare & Work (1957).

Teichoic acids. The procedures of Armstrong *et al.* (1958) and Davison & Baddiley (1963) were used to isolate the teichoic acids. Cell walls (0·25 g. wet wt.) were treated as described by these authors and the purified material containing teichoic acid hydrolysed in 4 N-HCl for 6 hr at 100° (Elliott, 1962). Ribitol and glycerol were separated by chromatography on Whatman No. 1 paper by using the solvent system of Armstrong *et al.* (1958), and were detected by the method of Bean & Porter (1959).

Sugars. Washed organisms (0·75 g. wet wt.) were suspended in 5 ml. of 2 N-sulphuric acid and treated as described by Davis & Baird-Parker (1959). Sugars

were separated by single-dimension chromatography on Whatman No. 1 filter paper by the solvent system of Colombo *et al.* (1960). Sugars were detected by using Roy's (1960) double spray technique.

Amino sugars. Washed organisms (0·75 g. wet wt.) were suspended in 5 ml. of 5 N-HCl and incubated for 2 days at 37°; the HCl was removed under vacuum in a vacuum desiccator containing concentrated H_2SO_4 and NaOH pellets (Salton, 1959). The residues were dissolved in 3 ml. of 10% (v/v) isopropanol in water and several drops of sp.gr. 0·880 ammonia added to neutralize remaining acid. After filtration through no. 4 porosity sintered glass filters, the filtrates were evaporated to dryness on a steam-bath and the residues dissolved in 0·3 ml. of 10% (v/v) isopropanol in water. The amino sugars were separated by chromatography on Whatman No. 1 filter paper by using the solvent system of Heyworth, Perkins & Walker (1961) and detected by using Elson & Morgan's (1933) spray.

Peptides and amino acids extractable by acetic acid. Acetic acid extracts of whole organisms were shown by Mattick *et al.* (1956) to be of use in identifying bacteria. The procedure was to suspend 0·5 g. wet weight washed organisms in 5 ml. of 10% (v/v) glacial acetic acid and to leave at room temperature for 1–2 hr with occasional shaking. The extracts were centrifuged at 10,000*g* to remove solids and the supernatant fluids evaporated to dryness in a rotary vacuum evaporator; the residues were finally suspended in 0·3 ml. volumes of 10% (v/v) isopropanol in water. The amino acids and low molecular weight peptides were separated by chromatography on Whatman No. 1 paper by using the solvent system of Davis & Freer (1960) and detected with the ninhydrin + collidine spray used for amino acids.

Electrophoretic separation of cell proteins. Starch gels (Smithies, 1955) were prepared by dissolving 15% (w/v) Connaught Brand hydrolysed starch in a semi-discontinuous buffer system containing 90% (v/v) tris + citrate buffer (pH 8·68) and 10% (v/v) borate buffer (pH 8·2); both buffers used were described by Poulik (1957) but LiOH replaced the NaOH specified in the original borate buffer (Mr A. L. Ogden, personal communication). The concentrated cell-content fractions were applied to strips of Whatman No. 17 filter papers (1·5 cm. × 6 mm.) and inserted 30 mm. from the cathode end of a gel 190 mm. long. A potential of 440 V and a current of 30 mA was applied along the gel, and electrophoresis continued until the moving boundary had migrated 80 mm. past the inserts. Protein bands were developed by staining slices of gel for 1 min. in a saturated solution of naphthalene black in methanol + water + glacial acetic acid (5 + 5 + 1 by vol.) followed by decolorization in several washes of the same solvent.

RESULTS

Preliminary examination of the morphology and catalase production of the organisms received showed that some had been incorrectly classified. Amongst these were three plasma-clotting strains of *Streptococcus faecalis* which had been sent to the author as coagulase-positive staphylococci and two other strains of *S. faecalis*, one of which had been identified as a coagulase-negative staphylococcus while the other had been named *Micrococcus freudenreichii*. Other wrongly classified organisms included several corynebacteria and unidentified Gram-negative rods; a strain of *Serratia marcescens* was received as a culture of *M. roseus*; a yeast was

received labelled as a micrococcus as were two strains of *Aerococcus* (*Pediococcus*) *viridans*. Several Gram-positive and catalase-positive cocci were also found to have been mis-classified as regards their genus or species.

Classification of organisms according to Baird-Parker (1963)

Five hundred and sixty-four of the cultures studied were Gram-positive catalase-positive cocci. With the exception of the pink micrococci, representatives of which had not been previously studied, almost all of the strains could be classified into the Baird-Parker (1963) groups and subgroups. To accommodate these pink pigmented organisms, a further subgroup, *Micrococcus* subgroup 8, was introduced. Characters of the fourteen subgroups are shown in Table 2.

Group 1 (*Staphylococcus Rosenbach*)

Organisms placed in this group grew and produced acid from glucose when incubated under anaerobic conditions in a mineral salts + yeast-extract medium containing bromocresol purple as pH indicator (Baird-Parker, 1963). With two exceptions, the morphological and physiological characters of the 180 cultures classifiable into this group agreed with previously studied cultures; i.e. they were usually small cocci (0·5–1·5 μ) growing as irregular clusters and producing on agar smooth convex colonies pigmented white, yellow or orange; their physiological characters included growth usually at 45° and sometimes at 10°. They also usually hydrolysed hippurate, proteins, fats and Tweens, and produced acid from galactose, glucose, glycerol, lactose, maltose, mannose and mannitol but not from arabinose, cellobiose, inositol, raffinose or xylose. Other characters included the ability of most strains to reduce nitrate beyond nitrite, and inability to hydrolyse aesculin or to grow on a medium containing ammonium phosphate as sole nitrogen source.

Characters of the subgroups within this group 1 also agreed closely with previous results. Exceptions were two otherwise typical subgroup II organisms which did not form acid from maltose and eight cultures which were acetoin-negative; four of the latter could be classified as acetoin-negative variants of subgroup V or VI. Of the four remaining acetoin-negative organisms only two possessed characters typical of staphylococci. One was coagulase-negative but similar to the coagulase-positive subgroup I organisms in the production of phosphatase and the formation of acid from mannitol in presence of air. However, it differed from sub-group I organisms by not coagulating human or rabbit plasma or forming acid from mannitol anaerobically. The other strain was phosphatase-negative and, of the sugar series tested, produced acid only from galactose, glucose and lactose.

The two apparently atypical staphylococci, both members of Abd-El-Malek and Gibson's group IIIB (NCTC 7567, 7568) appeared to be cocci when first examined. Characters which made me suspect that these were not staphylococci were: good growth on a medium containing ammonium phosphate as sole nitrogen source, hydrolysis of aesculin but not protein, fat, Tweens or hippurate, and formation of acid from a wide range of carbohydrates, including arabinose, cellobiose and xylose. On further examination of the cell morphology of these two organisms it was found that impression preparations of cultures grown for 4 hr on nutrient agar contained mainly short paired rods and that organisms examined in the electron microscope were

Table 2. *Complete characters of subgroups*

	(Group 1) *Staphylococcus* [Rosenbach] subgroups						(Group 2) *Micrococcus* [Cohn] subgroups								(Group 3) *Sarcina* [Good-sir]	*Aerococcus* [Williams, Hirsch & Cowan]
	I	II	III	IV	V	VI	1	2	3	4	5	6	7	8		
Colony																
(1) Nodular irregular edge	0	0	0	0	0	0	0	2	0	0	1	0	0	0	0	0
(2) Granular surface, regular edge	0	0	0	0	0	(8)	4 (4)	(22)	2 (10)	0	3 (9)	0	15 (25)	20 (30)	74 (22)	(63)
(3) Smooth convex regular edge	100*	100	100	100	100	92	92	76	88	100	86†	100	62	50	4	37
Pigment																
(1) White	10	97	100	75	75	68	63	93	84	100	75	34	18	0	10	5
(2) Yellow	0	0	0	25	17	28	37	0	8	0	17 (2)	0	82‡	0	70§ (2)	0
(3) Orange	90	0	0	0	8	(4)	0	2 (5)	(6)	0	3 (3)	66	0	0	4	0
(4) Red	0	0	0	0	0	0	0	0	0	0	0	0	0	100	14	0
(5) Violet	0	3‖	0	0	0	0	0	0	0	0	0	0	0	0	0	0
(6) Translucent	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	95
Appearance in broth																
(1) Fine deposit	97	71	100	100	100	96	42	41	73	25	19	100	54	70	26	68
(2) Granular deposit	0	(3)	0	0	0	(4)	13	2 (2)	8 (3)	0	3 (5)	0	13 (5)	10 (10)	12 (58)	16 (16)
(3) Mucoid deposit	(3)¶	(26)	0	0	0	0	21 (25)	12 (41)	6 (8)	(75)	32 (41)	0	10 (18)	(10)	2 (2)	0
Size																
$< 1\ \mu$	100	77	100	100	34	44	25	5	43	50	6	0	26	10	6	0
$1–1{\cdot}5\ \mu$	0	23	0	0	66	56	75	90	57	50	93	100	72	70	32	100
$> 1{\cdot}5\ \mu$	0	0	0	0	0	0	0	5	0	0	1	0	2	20	62	0
Catalase	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	0
Glucose																
Acid production under aerobic conditions	100	100	100	100	100	100	100	100	100	100	98 (2)	100	(10)	(60)	22 (16)	100
Glucose																
Acid production under anaerobic conditions	100	100	100	100	100	100	0	0	0	0	0	0	0	0	0	100
Coagulase production	100**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Phosphatase production	100	100	100	0	0	0	0	0	(1)	0	(1)	100	(13)	0	(30)	0
Terminal pH value in glucose	4·4–5·0 (av. 4·6)	4·2–5·1 (av. 4·5)	5·2	4·5–5·6 (av. 4·8)	4·5–5·3 (av. 4·6)	4·2–5·8 (av. 4·6)	4·4–5·6 (av. 4·9)	4·5–5·4 (av. 4·9)	4·5–5·8 (av. 4·9)	4·6–5·7 (av. 5·0)	4·9–5·9 (av. 5·3)	4·7–5·5 (av. 4·9)	6·4 8·2 (av. 6·9)	5·2–6·5 (av. 6·2)	4·6 8·4 (av. 6·3)	5·0 5·6 (av. 5·3)
Acetoin production	98 (2)	97 (3)	0	100	92	75 (18)	100	98 (2)	98	75 (25)	0	0	(9)	(10)	4 (6)	0

Table 2 (*cont.*)

Aerobic acid production from:																
Arabinose	0	0	0	0	0	0	0	0	0	100	6 (2)	78 (11)	0	0	8	0
Cellobiose	0	0	0	0	0	0	0	0	0	0	4 (2)	44	0	(10)	4 (2)	(16)
Dextrin	0	0	0	0	(8)	0	0	(2)	0	0	1 (1)	0	0	(10)	(2)	74
Galactose	54 (5)	71	100	100	67 (16)	22	0	60 (5)	52	100	78 (4)	67	0	(10)	12	95
Glycerol	100	100	0	100	100	100	75 (4)	95	98	100	96 (1)	100	(4)	10 (10)	24 (4)	100
Inositol	0	0	0	0	0	0	0	0	0	75	21 (3)	78	0	0	2	5
Lactose	92 (1)	71	100	0	100	62	0	98 (2)	57 (1)	100	75 (1)	33	0	0	12 (2)	94
Maltose	99 (1)	97	0	50	100	65	82 (4)	100	95 (1)	75	85 (2)	100	1 (9)	(10)	12 (14)	94
Mannose	100	55	(100)	50	17	44	4	5	22 (2)	50	63 (7)	63	0	(10)	10 (4)	100
Mannitol	96 (1)	0	0	0	0	96 (4)	0	0	98 (2)	50	80	100	0	(10)	4 (2)	56
Raffinose	0	0	0	0	0	0	0	0	0	0	2 (1)	0	0	0	0	10
Rhamnose	0	0	0	0	0	0	0	0	0	0	5 (1)	0	0	(10)	8	0
Salicin	0	0	0	0	0	0	4	0	0	0	3	33	0	0	0	10 (20)
Xylose	0	0	0	0	0	0	4 (4)	0	0	100	85 (2)	33	(2)	10 (10)	8 (2)	0
Hydrolysis of:																
Aesculin	3 (10)	(3)	0	(25)	8	(4)	25 (9)	(5)	8 (6)	75	44 (8)	89 (11)	0	(10)	2 (2)	56
Hippurate	99 (1)	87 (3)	100	100	92 (8)	100	67 (8)	95	94	100	86 (2)	89	23	30	12	100
Gelatin	63 (5)	68	100	50	25 (8)	21 (11)	44 (4)	41 (2)	24 (3)	25	25 (1)	78	84	90 (10)	90 (2)	0
Clearing of:																
Casein	91 (1)	61 (29)	100	25 (25)	42 (8)	43 (36)	20 (8)	5 (12)	24 (27)	0	8 (11)	33 (44)	31 (4)	0	14 (18)	0
Egg yolk	92 (2)	68	100	50	17 (17)	61 (11)	21	(5)	14 (1)	0	7 (4)	(11)	16 (14)	0	12 (18)	0
Ammonia from arginine broth	100	81 (6)	100	75	42 (8)	89	50 (18)	64 (7)	90 (6)	100	48 (20)	78 (22)	4 (9)	0	20 (6)	0
Nitrate																
No reduction	0	16	0	0	0	4	17	32	48	0	16	0	85	50	83	100
Reduced to nitrite	6	13	0	0	14	21 (14)	8	30	14	25	61 (1)	56	4 (9)	30 (10)	5	0
Reduced beyond nitrite	94	71	100	100	86	61	75	38	38	50	22	44	2	10	12	0
Lard hydrolysis	96	100	0	50	58	64 (7)	42	75 (2)	66 (8)	25	34 (2)	11	97 (7)	0	30 (12)	0
Butter hydrolysis	96	100	0	50	58	64 (11)	42	76	68 (6)	25	34 (3)	11	23 (14)	0	32 (14)	0
Tween 20 hydrolysis	98	100	100	50	58	96	27	35 (5)	46 (1)	0	0	11	54 (2)	100	50 (8)	0
Tween 40 hydrolysis	93 (5)	100	100	50	50 (8)	96	14	24 (3)	41 (3)	0	0	11	63 (4)	100	64 (2)	0
Tween 60 hydrolysis	91 (7)	100	100	50	33 (25)	89	14 (4)	24	43 (3)	0	0	11	58 (7)	90	52 (6)	0
Tween 80 hydrolysis	53 (18)	97	100	50	27 (17)	89	13 (4)	17	26 (1)	0	0	11	19 (7)	40	32 (6)	0
Ammonium phosphate used	0	(3)	0	0	0	0	21	24 (10)	41 (6)	0	38 (13)	89	19 (25)	10	22 (38)	0
Growth in the presence of no added NaCl	100	55 (45)	100	100	83 (17)	100	96 (4)	98 (2)	92 (8)	100	99 (1)	100	100	100	100	100
Growth in the presence of 5 % NaCl	99 (1)	100	100	100	92 (8)	100	88 (12)	98 (2)	95 (5)	100	100	100	88 (12)	30 (20)	90 (10)	95 (5)
Growth in the presence of 15 % NaCl	58 (27)	61 (10)	100	75	25 (33)	64 (14)	21 (4)	51 (20)	70 (11)	75 (25)	76 (9)	78 (11)	(2)	0	0	0
Growth at 10°	68 (14)	4 (11)	0	0	16	61 (8)	72 (8)	69 (11)	71 (7)	100	97	88 (12)	70 (13)	100	54 (2)	100
Growth at 45°	91 (1)	94	100	100	94	82	41 (4)	37 (5)	40 (5)	0	9 (9)	33	21 (2)	(10)	28 (2)	(21)
Total organisms	80	31	1	4	13	32	25	41	72	4	152	9	38	10	55	19

* % of isolates positive.
† Includes 6 cultures with crenated peripheries.
‡ Includes 2 cultures producing yellow and violet colonies (*S. flavocyaneus*).
§ Includes 1 culture producing yellow and violet colonies (*S. flavocyaneus*).
¶ Production of violet pigment on potato and glucose agar but not on nutrient agars.
‖ % of isolates weakly positive.
** Two cultures produced only bound coagulase (Duthie, 1954).

definitely rod-like. In their paper Abd-el-Malek & Gibson (1948) mentioned that they were heat-resistant (survived 60° for 30 min.), were unable to grow at 37° and formed colonies with a greenish-yellow pigment. These characters made me suspect that the two organisms were microbacteria. A culture of *Microbacterium lacticum* (NCDO 747) was examined and although it was morphologically distinguishable from Abd-el-Malek and Gibson's two strains it possessed very similar biochemical characters. The results of cell-wall analyses supported the exclusion of these organisms from the genus *Staphylococcus* (see discussion of results of cell analyses).

Group 2 (Micrococcus Cohn)

Group 2 contains cocci which were either unable to produce sufficient acid from glucose to change the indicator bromocresol purple or which grew and produced acid from glucose only in presence of air. They were generally much more variable in size than the staphylococci and formed tetrads as well as irregular clusters of cocci. Their colonies were either smooth and convex or slightly granular and were pigmented white, yellow, orange, red, brown or violet. Their growth in broth was usually either a mucoid or granular precipitate, in contrast to the fine deposit produced by staphylococci.

The eight subgroups differed in their carbohydrate metabolism. Subgroups 1–3 (Baird-Parker, 1963) possessed similar characters to the staphylococci in forming acetoin and an end pH value in glucose broth of generally below 5·0; subgroups 5 and 6 differed from these in attacking a wider range of carbohydrates with a higher end pH value in glucose broth without producing acetoin; subgroup 4 appeared to be intermediate. Subgroups 7 and 8 formed either no acid or only small amounts from glucose.

Some characters of these subgroups differed in some respects from those previously reported (Baird-Parker, 1963). Thus, 20–40 % of subgroups 1 and 2 hydrolysed one or more of the Tweens, whereas only weak activity was shown by one organism in the small collection studied previously. Also, a larger number of the members of these subgroups grew at 10° but not at 45°. With the exception of four organisms, the 72 cultures classifiable in *Micrococcus* subgroup 3 could be placed in one or other of the three types previously defined within this subgroup (Baird-Parker, 1963). One of the acetoin-negative organisms was placed in this subgroup rather than with the acetoin-negative subgroups since it hydrolysed Tweens and did not utilize arabinose, xylose or aesculin. The characters of subgroup 5 were mainly the same as those found previously, but the subgroup 6 members, the phosphatase-positive micrococci, were different in that most strains did not hydrolyse Tweens. The pink micrococci placed in subgroup 8 were very similar morphologically and biochemically to subgroup 7 members, but generally differed from the latter in producing more acid from glucose and utilizing a wider range of carbohydrates (see Table 2).

Group 3 (Sarcina Goodsir)

Members of this group grew as regular packets of eight or more cocci. Their physiological characters showed quite wide variations but the majority were similar to *Micrococcus* subgroup 7 or 8.

Relationship of my classification to previous taxonomic groupings

One hundred and ninety-four named cultures were examined and, of these, 178 were established to be Gram-positive catalase-positive cocci. These belonged to

Table 3. *Classification of named species, groups and subgroups according to classification of Baird-Parker* (1963)

Classification according to Baird-Parker (1963)	Original classification
Staphylococcus	
Subgroup I	*S. aureus* (24)*
Subgroup II	*S. albus* (2), *S. epidermidis*, *S. saprophyticus* (2)[1], *M. aurantiacus*, *M. viola-gabriellae* (*S. saprophyticus*[1], *S. epidermidis*[2])
Subgroup III	*M. hyicus* (*S. lactis*[1])
Subgroup IV	*S. epidermidis*
Subgroup V	*S. albus*, *S. epidermidis*[2], *S. saprophyticus* (2)[1], *M. cerolyticus* (*M. conglo-meratus*[2])
Subgroup VI	*S. albus* (*S. lactis*[1]), *S. epidermidis* (2)[2], *S. saprophyticus* (2)[1]
Micrococcus	
Subgroup 1	*S. epidermidis*, *S. saprophyticus* (3)[1]
Subgroup 2	*S. saprophyticus* (*S. epidermidis*[2])
Subgroup 3	*M. candicans* (2) (*S. saprophyticus*[1], *S. epidermidis*[2]), *M. candidus* (2) (*S. saprophyticus*[1], *S. epidermidis*[2]), *S. albus* (2) (*S. saprophyticus*[1], *S. epidermidis*[2])
Subgroup 5	*M. aurantiacus* (*S. lactis*[1]), *M. conglomeratus* (2)[2], *M. euryhalis* (*M. varians*[2]), *M. varians* (10)[2], *S. lactis* (3)[1], *Sarc. aurantiaca* (*S. lactis*[1]), (*M. conglomeratus*[2]), *Sarc. lutea* (2) (*S. lactis*[1], *M. luteus*[2]). Abd-el-Malek & Gibson's (1948) group IIIA (4) (*S. lactis*[2]); Pohja's subgroups e, i, l, g, d, k, c.
Subgroup 6	*M. varians* (3)[2], *S. lactis*[1], Pohja's subgroup f
Subgroup 7	*M. agilis* (*S. afermentans*[1]), *M. flavus* (5) (*M. luteus* (5)[2]), *M. luteus* (8)[2], *M. lysodeikticus* (4) (*S. afermentans* (3)[1], *M. luteus*[2]), *M. sodonensis* (*M. luteus*[2]), *S. afermentans*[1], *S. flavocyaneus* (3) (*S. afermentans*[1], *M. luteus* (2)[2]), *Sarc. flava* (2) (*S. afermentans*[1], *M. luteus*[2]), *Sarc. lutea* (*M. luteus*[2]), Abd-el-Malek & Gibson's group IIB (*S. afermentans*[1]), Abd-el-Malek & Gibson's group IIC (*S. afermentans*[1])
Subgroup 8	*M. agilis* (2) (*S. afermentans* (2)[1]), *M. roseus* (2) (*S. roseus* (2)[1]), Pohja's subgroup h
Sarcina	*Sarc. aurantiaca* (*M. luteus*[2]), *Sarc. citrea* (*M. luteus*[2]), *Sarc. flava* (2) (*S. afermentans*[1], *M. luteus*[2]), *Sarc. lutea* (4) (*S. afermentans* (2)[1], *M. luteus* (2)[1]), *Sarc. marginata* (*M. luteus*[2]), *Sarc. variabilis* (*M. luteus*[2]), *M. corralinus* (*S. roseus*[1]), *M. citreus* (*M. luteus*[2]) *M. flavus* (4) *M. luteus* (4)[2], *M. flava-roseus* (*S. roseus*[1]), *M. luteus* (8)[2], *M. roseus* (4) (*S. roseus* (4)[1]), *M. rubens* (*S. roseus*[1])

Organisms possessing characters that were very different from their original descriptions: *Staphylococcus* subgroup I: *S. epidermidis* (7)[2], *S. epidermidis-albus* (1) (*S. aureus*[1]), *S. lactis*[1], *M. caseolyticus* (*S. aureus*[1]); subgroup II: *S. aureus*; *Micrococcus* subgroup 1: *M. cremoris-viscosi* (*S. lactis*[2]), *M. citreus*; subgroup 2: *M. caseolyticus*; subgroup 3: *M. luteus* (2) (*S. saprophyticus*[1], *M. conglomeratus*[2]); subgroup 5: *Sarc. ventriculi* (*M. conglomeratus*[2]); subgroup 6: *S. aureus*; subgroup 7: *M. candicans* (*S. afermentans*[1]), *M. freudenreichii* (*S. afermentans*[1]), *M. papyro-absorbens* (*S. lactis*[1]); subgroup 8: *M. varians*.

Key:

(24)* = no. of strains studied.

(*S. saprophyticus*[1]) Reclassified according to the classification of Shaw *et al.* (1951) by either the National Collection of Type Cultures (Dr S. T. Cowan) or the National Collection of Dairy Organisms (Dr E. I. Garvie).

(*S. epidermidis*[2]). Reclassified according to the classification of Kocur & Martinec (1962) by Dr M. Kocur of J. E. Purkyne University, Brno, Czechoslovakia.

9 named species of staphylococci, 25 of micrococci, 9 of sarcinas and 12 to groups or subgroups within these genera. Table 3 shows the relationship of these taxa to each other and to the author's groups and subgroups.

The majority of the named species and groups were received through the generosity of Dr S. T. Cowan (Curator of the National Collection of Type Cultures), Dr Ellen I. Garvie (Curator of the National Collection of Dairy Organisms) and Dr M. Kocur (Curator of the Culture Collection of J. E. Purkyne University, Brno, Czechoslovakia). The first two of these sources have reclassified the cultures in their collections according to the scheme of Shaw *et al.* (1951) and the last according to that of Kocur & Martinec (1962). Therefore, to indicate in Table 3 where organisms have been renamed by one or other of these sources, the original name is given followed by the new name in parentheses with a superscript referring to the source of this name. Where the same strain was received from more than one source only one is recorded in Table 3. Also, to avoid confusion, strains whose characters did not in any way correspond to their published descriptions are listed at the end of the Table.

Relationship of classification to habitat

Three hundred and twelve coagulase-negative, Gram-positive catalase-positive cocci were received as isolates from known sources. Table 4 shows the distribution of isolates from habitats from which ten or more organisms were received. For ease of interpretation, the numbers of each subgroup are expressed as a percentage of the total number of isolates from a particular source.

Table 4. *Percentage distribution of coagulase-negative Gram-positive catalase-positive cocci from different sources*

	Sub-group	Human		Air	Soil	Dairy products	Fermented Meats	Marine	Bacon	Brine	Pork
		Nose	Skin								
Staphylococcus	II	62	40	4	0	0	0	0	0	0	0
	IV	0	8	0	0	0	0	10	0	0	0
	V	5	4	8	0	0	0	0	0	0	0
	VI	0	32	6	16	0	0	20	2	0	4
Micrococcus	1	0	4	4	8	12	0	5	8	0	20
	2	18	4	14	0	19	0	5	4	8	32
	3	0	4	46	44	12	0	10	0	4	11
	4	0	0	0	0	0	0	0	4	0	2
	5	5	4	4	16	19	70	20	82	88	27
	6	0	0	2	0	7	10	10	0	0	0
	7	5	0	0	16	0	10	5	0	0	4
	8	0	0	0	0	0	10	5	0	0	0
Sarcina		5	0	12	16	31	0	10	0	0	0
Total organisms		21	25	48	12	16	10	19	46	55	44

Staphylococci predominated among the Gram-positive catalase-positive cocci obtained from human skin and nasal surfaces, whereas micrococci occurred more frequently amongst isolates from air, soil, meat and dairy produce. The predominance of *Staphylococcus* subgroup II and VI organisms on human skin confirms

previous results (Baird-Parker, 1962), as also does the finding that members of *Micrococcus* subgroup 3 are the main micrococci occurring in air samples (Baird-Parker, unpublished). The only coagulase-negative staphylococci received from marine sources were isolates from the skin of freshly-caught cod and herring.

Relationship of classification to chemical components of the cell

Forty-six strains, representative of my groups and subgroups, were examined for chemical components of the cells. Three main cell fractions were examined: whole organisms for sugars, amino sugars and acetic acid-extractable small peptides and amino acids; cell walls for amino acids and teichoic acids; cell contents for proteins.

The cell-wall mucopeptide of all strains contained alanine, glutamic acid and lysine, together with, in most strains varying amounts of aspartic acid, glycine and serine, and in some strains small amounts of leucine, threonine and valine. The results (Table 5) show only small differences in the overall peptide composition of the subgroups. There does, however, appear to be a simplification in the amino acid composition of the peptides as one proceeds from *Staphylococcus* subgroups I and II containing up to 9 amino acid components, through the other staphylococcus and micrococcus subgroups to *Micrococcus* subgroup 8 containing only 4 or 5 amino acids.

Other than ribose, glucose was the only sugar detected in staphylococci and *Micrococcus* subgroups 1–4, whereas glucose, galactose and/or mannose were present in many of the strains belonging to *Micrococcus* subgroups 5–8 and *Sarcina* (Table 5). Thus, with respect to sugars, there was an increase in complexity from *Staphylococcus* subgroup I containing little or no sugar other than ribose to *Micrococcus* subgroup 8 containing ribose and quite large amounts of glucose, galactose and mannose. Glucosamine and muramic acid were present in all the strains examined, with galactosamine in most strains. Variable amounts of teichoic acids were present in the cell walls of most strains but were not detected in walls of either *Micrococcus* subgroup 7 and 8. Glycerol-containing teichoic acids were most commonly found, although these were replaced by ribitol-teichoic acids in *Staphylococcus* subgroup I. Small amounts of ribitol were detected together with glycerol in two members of *Micrococcus* subgroup 2 (Table 5).

Differences in acetic acid-extractable amino acids and peptides were determined by comparing the patterns of ninhydrin-staining spots on two-dimensional chromatograms; no attempt was made to determine the position of individual amino acids by markers or the structure of the peptides by hydrolysis. Two main patterns were distinguishable. One was typical of most staphylococci, members of *Micrococcus* subgroups 1–3 and some members of *Micrococcus* subgroups 5 and 6. The other was shown by the majority of strains of *Micrococcus* subgroup 5 and all strains belonging to *Micrococcus* subgroups 7 and 8 and *Sarcina*.

Electrophoresis of proteins from cell contents yielded a variety of distinct patterns of protein bands. Some of these patterns appeared to be characteristic of certain subgroups, e.g. *Staphylococcus* subgroups II, III, IV and V and *Micrococcus* subgroup 8. Other subgroups, however, gave a variety of patterns within a subgroup, e.g. *Staphylococcus* subgroups I and VI.

Table 5. *Cell and cell-wall components of staphylococci and micrococci*

		Arabinose	Galactose	Glucose	Mannose	Ribose	Rhamnose	Alanine	Aspartic acid	Diaminopimelic acid	Glutamic acid	Glycine	Leucine	Lysine	Phenylalanine	Serine	Threonine	Valine	Galactosamine	Glucosamine	Muramic acid	Glycerol-teiochic acid	Ribitol-teiochic acid	No. of cultures
Staphylococcus																								
Subgroup	I	–	–	– or tr	–	+	–	+	+ or tr	–	+	+	+	+	–	+	+ 1	+ 1	–	+	+	– or tr	+	3
	II	–	–	+	–	+	–	+	+ 1	–	+	+	+ 1	+	–	+	+ 1	+ 1	+ 2	+	+ or tr	+	–	6
	III	–	–	+ 1a	–	+	–	+	– 2	–	+	–	–	+	–	+	–	–	–	+	+	+ ?	–	3
	IV	–	–	+ 2	–	+	–	+	+ 1	–	+	+	–	+	–	+	–	–	–	+	+	+	–	3
	V	–	–	+ 1	–	+	–	+	+ 1	–	+	+	–	+	–	+ or +	–	–	+ or tr	+	+	+ 1	–	3
	VI	–	–	+ or +	–	+	–	+	+ 4	–	+	+	–	+	–	+	–	–	+ 5	+	+	+ 5	–	6
Micrococcus																								
Subgroup	1	–	+	+ or +	+ 1	+	–	+	+	–	+	+	–	+	–	+	–	–	+	+	+	+ 2	– 2	6
	2	–	–	+ or +	–	+	–	+	+ 1	–	+	+	–	+	–	+	–	–	+ 2	+	+	+ 2	+ 2	7
	3	–	–	+ or +	–	+	–	+	+ 1	–	+	+	–	+	–	+	–	–	+ 2	+	+	+ 2	– 2	3
	4	–	+ 2	+	+ 1	+	–	+	–	–	+	+	–	+	–	+ or +	+ 1	–	NR	NR	NR	NR	NR	1
	5	–	+ 1	+	+ 1	+	–	+	+ 1	–	+	+	–	+	–	+	+	–	+ 2	+	+	+	–	3
	6	–	–	+	+ 2	+	–	+	–	–	+	+	–	+	–	+	+ 1	–	+	+	+	tr	–	2
	7	–	–	+	+	+	–	+	+ 1	–	+	+	–	+	–	+	–	–	tr	+	+	–	–	3
	8	–	–	+	+	+	–	+	or tr	–	+	– or tr	–	+	–	+	–	–	–	+	+	–	–	3
Sarcina		–	–	+	+ 1	+	–	+	+	–	+	+	–	+	–	–	–	–	tr	+	+	–	–	2

+ + = present in large amounts; + = present in small amounts; tr = present in trace amounts; NR = no result; + 1a = no. of strains positive.

DISCUSSION

A detailed examination was made of the morphology and physiological characters of 607 cocci isolated from different sources and parts of the world. Before this work began, each strain was examined to determine its morphology, stainability by Gram's method and catalase production; this was done to exclude strains which did not correspond to the description: Gram-positive and catalase-positive coccus dividing in more than one plane. One or more of these characters was absent from forty-three of the strains examined. From this examination, there would appear to be two main sources of error in identifying Gram-positive catalase-positive cocci. Thus, cocci can be confused with Gram-positive and Gram-negative rods, with short chains of cocci and with budding yeasts, while citrate-utilizing plasma-clotting strains of faecal streptococci may be identified as coagulase-positive staphylococci. For several reasons it is considered that several of the established *Micrococcus* species are not true members of the Micrococcaceae. Thus, *M. denitrificans*, which is Gram-negative and forms rods in young cultures (Verhoeven, 1957), has a cell-wall mucopeptide which contains a wide range of amino acids characteristic of Gram-negative genera (Baird-Parker, unpublished). In a recent paper Pleva & Kocur (1963), regarded *M. denitrificans* as a valid member of the genus *Micrococcus* although they found that it showed definite rods when examined in the electron microscope. The related species *M. halodenitrificans* is also Gram-negative (Robinson & Gibbons, 1952) and also possesses a characteristic Gram-negative cell wall with respect to both its high lipoprotein content (Smithies, Gibbons & Bayley, 1955) and its wide range of amino acids (Takahashi & Gibbons, 1959). It is also probable that *M. radiodurans* should be reclassified since its cell wall contains lipoprotein and a wide range of amino acids (Work, 1964); the guanine: cytosine ratio of its nucleic acid is also more like to that of the Gram-negative genera (Dr B. Moseley, personal communication). The probable identity of Abd-el-Malek & Gibson's group IIIB with the genus *Microbacterium* on the grounds of morphology and physiological characters has already been mentioned; further evidence for excluding these organisms from the Micrococcaceae is the presence of diaminopimelic acid in their cell walls (Baird-Parker, unpublished).

The non-capsulate cocci which form short chains and grow on salt-containing media, i.e. the faecal streptococci, are frequently confused with coagulase-positive and -negative staphylococci. They are most readily distinguished from the cluster-forming members of the Micrococcaceae by the catalase test (Isaacs & Scouller, 1948; Evans, Buettner & Niven, 1952; Brown & Evans, 1963) and although there are a few reports of catalase-positive faecal streptococci (Langston, Guttierez & Bouma, 1960) and of catalase-negative staphylococci (Lucas & Seeley, 1955; Jensen, 1963; Solomon & San Clemente, 1963) these reports are rare. Another group of organisms which may be confused with the staphylococci are the aerococci. The latter were originally reported to be catalase-negative but they may decompose hydrogen peroxide under certain conditions (Günther & White, 1961). Also, Clausen (1964) reported the isolation of an *Aerococcus* sp. (*A. catalasicus*) which was nitratase-positive and catalase-positive. It may be necessary, therefore, to use tests other than catalase formation to distinguish these organisms from staphylococci and micrococci. They can be distinguished from micrococci by their fermentative meta-

bolism (Cowan & Steel, 1961) and from the staphylococci by their failure to hydrolyse arginine, proteins, fats or Tweens (Table 2). Aerococci can also be distinguished from staphylococci and from pediococci, which only form catalase on low-glucose media (Felton, Evans & Niven, 1953), by their formation of peroxide. The production of peroxide may be detected by streaking a suspected aerococcus across a heart-infusion agar plate seeded with *Staphylococcus aureus* and incubating at 20° for 3–4 days. The production of peroxide is indicated by inhibition of the staphylococcus in a wide zone surrounding the aerococcus growth; a control plate containing catalase should also be set up, to exclude inhibition due to antibiotic production (Baird-Parker, unpublished).

CLASSIFICATION

The majority of organisms which satisfied the criteria of morphology and catalase production could be classified into the groups and subgroups previously defined (Baird-Parker, 1963). Excluding the pink pigmented micrococci, over 96% of the remaining organisms could be classified into my thirteen subgroups. A further 3% of the organisms differed from previously defined subgroups in one main character only and were therefore considered to be variants of these subgroups. Only two organisms, both staphylococci, were sufficiently different from those previously studied to suggest that they should be placed in further subgroups.

The results obtained support the separation of staphylococci from micrococci on ability to grow and produce acid from glucose in the absence of air. This character separates staphylococci as a group of potential pathogens or commensals which are characteristic of animal and human surfaces, from micrococci which are a group of mainly free-living saprophytes which are nutritionally less exacting and morphologically and biochemically more variable than the staphylococci. Contrary to my previous conclusions (Baird-Parker, 1963), to place the packet-forming cocci in the genus *Sarcina* (group 3) could not be justified. This reversal of my conclusions has resulted from a study of the morphology of named *Sarcina* spp. grown under a variety of conditions. When this was done, many of the named *Sarcina* spp. lost their ability to form packets. Of 55 organisms growing as regular packets of cocci on one or more media, only 20 invariably formed packets; under none of the combinations of media and temperature tested would they all form packets. It was further found that the pink-pigmented packet-forming cocci were indistinguishable biochemically and on their chemical constituents from the pink-pigmented micrococci of *Micrococcus* subgroup 8. Therefore, in agreement with Shaw *et al.* (1951) and Kocur & Martinec (1962) it must be concluded that the ability to form cubical packets of cocci is characteristic of certain micrococci and that these, like the tetrad formers, belong mainly to *Micrococcus* subgroups 7 and 8. In the following sections of this paper, the packet-forming cocci will be discussed with the micrococci.

Separation of the Gram-positive and catalase-positive cocci into two groups or genera is supported by the results of Thomas & Schuhardt (1964), who examined the effect of lysostaphin (Schindler & Schuhardt, 1964) on these organisms. They found that staphylococci (defined on ability to grow and produce acid from glucose anaerobically) were lysed by this substance but that the micrococci and sarcinas remained intact.

Group 1 (Staphylococcus Rosenbach)

Subgroup I (Staphylococcus aureus)

The coagulase test is frequently used as sole criterion for recognizing *Staphylococcus aureus*. Ability to clot mammalian blood plasma is not, however, specific for *S. aureus* and by no means all members of this species by clot plasma. Amongst Gram-negative species which may clot plasma, by citrate utilization or coagulase-like activity, are strains of *Pseudomonas aeruginosa* (Frédéricq, 1941), *Serratia marcescens* (Frédéricq, 1946) and *Pasteurella pestis* (Eisler, 1961), and amongst Gram-positive organisms are actinomycetes (Frédéricq, 1946) and strains of *Streptococcus faecalis* (Evans *et al.* 1952). The inability of *S. aureus* to clot plasma may be due to use of unsuitable plasma, the absence of bound and/or free coagulase (Duthie, 1954), or excessive production of fibrinolysin or a coagulase-destroying factor (Lominski, Smith & Morrison, 1953; Munch-Peterson, 1961). The need to control the coagulase test was shown by the finding that seven strains designated as *Staphylococcus epidermidis* on inability to clot human blood plasma were, when tested in this laboratory, found to be typical strains of *S. aureus* which were able to clot rabbit plasma, ferment mannitol and to produce phosphatase; similarly a strain received as *S. epidermidis* had been misclassified because it produced bound coagulase (slide test) but not free coagulase (tube test). The inadvisability of relying solely on the coagulase test for the identification of *S. aureus* was also shown by the receipt of two diphtheroids and five strains of *S. faecalis* which had been classified by their isolators as strains of *S. aureus* because of ability to clot blood plasma. Amongst other strains of *S. aureus* which were examined and found to be of doubtful authenticity (see Table 3) was the propagating strain of phage type 73 (NCTC 8360). In a recent study of the physiological characters of the phage-propagating strains of *S. aureus*, Solomon & San Clemente (1963) reported that phage type 73 showed neither coagulase nor phosphatase activity. Dr I. Lominski (Dr K. J. Steel, personal communication) has shown that this organism will clot sheep plasma but not human or rabbit plasma. When I tested this organism it was coagulase-negative with rabbit plasma but phosphatase-positive, able to utilize glucose and mannitol only in the presence of air, to produce acid from arabinose and grow on a medium containing ammonium phosphate as sole nitrogen source. These characters make the organism unlikely to be a strain of *S. aureus*, and according to my work it should be classified in *Micrococcus* subgroup 6.

Anaerobic utilization of mannitol and the coagulase test were considered by Mossel (1962) to be the most useful criteria for distinguishing *Staphylococcus aureus* from other staphylococci and micrococci. He found that 95% of coagulase-positive strains from clinical and non-clinical sources fermented mannitol. However, ability to form acid from mannitol anaerobically may depend on the source of organism, since White, Rattray & Davidson (1963) reported that only 73% of bovine strains of *S. aureus* fermented mannitol. Also in a recent survey Cowan & Steel (1964) found that only 75% of the coagulase-positive staphylococci they tested were able to ferment mannitol under the conditions defined by Mossel (1962).

Our knowledge of variation in the physiological characters of *Staphylococcus aureus* indicates that a single character such as formation of coagulase cannot be solely relied upon for critical determination of this species. Therefore, before a

coagulase-negative strain is classified with coagulase-negative staphylococci several further tests should be made to exclude the possibility that it is a coagulase-negative variant of *S. aureus*. The best tests for this purpose are the determination of phosphatase production and mannitol fermentation. These tests when used in conjunction with the free and bound coagulase tests (Duthie, 1954) will clearly separate *S. aureus* from related staphylococci; positive recognition of this species is obtained when at least two of these tests are positive. Further confirmation of diagnosis can be obtained by determining phage typability (Parker, 1962), haemolysin pattern (Elek & Levy, 1950) and ability to grow in normal human serum (Fletcher, 1962).

Staphylococcus subgroups II and III

Members of these subgroups produce phosphatase and are distinguished from *Staphylococcus aureus* by not forming acid from mannitol or coagulating plasma; they are separated by acid production from maltose and acetoin from glucose (Table 3). Staphylococci belonging to subgroup III have only been isolated from pig skin while members of *Staphylococcus* subgroup II have been isolated from several sources, although their main habitats would appear to be human and animal surfaces.

It has been suggested that ability to produce phosphatase is associated with pathogenicity and the coagulase test (Barber & Kuper, 1951). However, the majority of coagulase-negative staphylococci from human sources are phosphatase producers (Baird-Parker, 1962; Jacobs, Willis & Goodburn, 1963). Coagulase-negative staphylococci may, however, be associated with, and cause, a number of infective processes (Pereira, 1962). Two coagulase-negative pathogens studied by me were both phosphatase producers; one was a member of *Staphylococcus* subgroup II, the other of *Staphylococcus* subgroup III. The subgroup II organism was a strain of *Micrococcus violagabriellae* Castellani. This organism was isolated by Castellani (Castellani, 1955) from a chronic skin infection occurring in a group of South American marines and was shown by him to give rise to this condition on re-inoculation. Its physiological characters were identical with the apparently non-pathogenic subgroup II staphylococci that were isolated from human skin, but it possessed the additional property of producing a characteristic violet pigment when grown on glucose- or potato-containing media (Castellani, 1955; Steel, 1964). It should be mentioned that the strain studied did not have the same characters as those reported for *M. violagabriellae* by Kocur & Martinec (1963*a*). Reclassification of *M. violagabriellae* in the genus *Staphylococcus* is in agreement with the results of Sneath (1960) and Kocur & Martinec (1963*a*).

The other phosphatase-producing coagulase-negative pathogen studied was a strain of *Micrococcus hyicus* Sompolinsky. This was reported by Sompolinsky (1953) to be the causative organism in an outbreak of contagious impetigo of swine. It possesses similar physiological characters to the subgroup III staphylococci previously isolated from pig skin (Baird-Parker, 1963) but was unable to utilize glycerol (Table 2). *M. hyicus* produces necrotic lesions when subcutaneously injected into mice (Jones, Deibel & Niven, 1963). I confirmed this observation but was unable to produce similar lesions with strains of *Staphylococcus* subgroup III isolated from pig skin. The physiological characters of *M. hyicus* were the same

as those reported by Jones *et al.* (1963), and in support of their conclusions it is suggested that this organism should be transferred to the genus *Staphylococcus*. According to Jacobs *et al.* (1963) potentially pathogenic staphylococci produce deoxyribonuclease as well as phosphatase; this is true of *M. hyicus* (Jones *et al.* 1963). However, deoxyribonuclease activity was shown by several subgroup II staphylococci which I isolated from normal skin and by a number of saprophytic micrococci belonging to subgroups 2, 5 and 7. The correlation between deoxyribonuclease production and potential pathogenicity therefore requires further investigation.

Staphylococcus subgroups IV, V and VI

Organisms received as strains of *Staphylococcus albus*, *S. epidermidis* and *S. saprophyticus* were classified in subgroups IV, V and VI (Table 4). *Staphylococcus* subgroups IV and V possess physiological characters which suggest a relationship with either subgroup II or VI. Thus some strains of *Staphylococcus* subgroups IV were only separated from some members of subgroup VI by their inability to form acid from mannitol and subgroup V members from subgroup II by absence of phosphatase activity. In future considerations of species it is probable that subgroups IV and V will be combined with subgroups VI and II respectively. They are at present retained in separate subgroups as proteins electrophoretically separated from cell contents of members of these subgroups are quite distinct.

Members of *Staphylococcus* subgroup VI are separated from those of subgroup I, II and III by their aerobic utilization of mannitol and the absence of coagulase and phosphatase. They are characteristic of human and animal skins and were found amongst isolates from the skin of freshly-caught fish. However, their presence on fish skin may have occurred through handling of the skin before sampling.

Group 2 (Micrococcus Cohn)

Micrococcus subgroups 1, 2, 3 and 4

These subgroups are physiologically closely related and form acetoin as an end product of glucose metabolism. Strains of *Staphylococcus epidermidis* and *S. saprophyticus* were classified in these subgroups as also were cultures originally labelled *S. albus*, *M. candicans* and *M. candidus* but which had been reclassified as either *S. epidermidis* or *S. saprophyticus* (Table 3). As mentioned previously (Baird-Parker, 1962, 1963) these subgroups contain organisms which are distinguished from staphylococci solely on their inability to use glucose under anaerobic conditions. It is with these organisms that the main controversy exists about separation of the Gram-positive catalase-positive cocci into two genera. Evidence can be cited which suggests classification with the staphylococci on the grounds of similar physiological characters and chemical composition, and with the micrococci on grounds of inability to grow anaerobically and to use glucose, and in a number of strains on the ability to produce acid from glucose in a medium containing ammonium phosphate as sole nitrogen source. Unfortunately, tests for anaerobic growth and glucose utilization are fairly critical and a result obtained by one method may not always agree with the result obtained by another method. Recently Cowan & Steel (1964) tested methods which have been used for distin-

guishing staphylococci from micrococci against a comprehensive collection of 87 strains of Gram-positive catalase-positive cocci. They found that 50 of these strains were classifiable as staphylococci by the Hugh & Leifson (1953) test but only 35 of these were classifiable as staphylococci by Baird-Parker's test. Three tests correlated well: these were Baird-Parker's test, the test of Evans, Bradford & Niven (1955), when the medium after incubation was below pH 5·0, and the ability to produce acid from glucose in a peptone medium incubated anaerobically.

A subcommittee set up by the International Association of Microbiological Societies is considering the methods for the separation of staphylococci from micrococci. At the first meeting of the subcommittee it was recommended that a standard test should be introduced to determine the anaerobic utilization of glucose and other carbohydrates by staphylococci. The method proposed was to inoculate growth from an actively growing culture into a tube of a semi-solid agar medium containing peptone + yeast extract + carbohydrate and bromocresol purple as pH indicator and to seal the tube with a layer of liquid paraffin. Tubes are incubated at 37° for 5 days. If, when glucose is present, acid is produced throughout the tube, the organism is a staphylococcus; but if no acid is formed, or acid is only formed at the top of the tube, the organism is a micrococcus.

Micrococcus subgroups 5 and 6

Members of these subgroups oxidize a wider range of carbohydrates than those of subgroups 1–4 and are unable to form acetoin from glucose; the two subgroups are separated by phosphatase production. Subgroup 5 contains a large group of closely related organisms. These have been given a variety of specific names (see Table 3). Shaw *et al.* (1951) and Abd-el-Malek & Gibson (1948) placed these organisms in *Staphyloccus lactis* and group IIIA respectively. Two of the most recent detailed classifications of the micrococci by Pohja (Pohja, 1960; Pohja & Gyllenberg, 1962) and by Kocur & Martinec (1962) have attempted to subdivide these micrococci into a number of subgroups or species. Pohja (1960) used 'numerical taxonomy' (Sneath, 1962) for his studies and subdivided his acetoin-negative micrococci into eleven subgroups; eight of these corresponded to my subgroups 5 and 6; the remainder were either halophilic or pink-pigmented. Primary division of these eight subgroups was based on salt tolerance and further division on optimum salt concentration and the production of acid from arabinose, and urease, gelatinase and nitratase activity. In a further paper Pohja & Gyllenberg (1962) recognized six subgroups of halotolerant micrococci and distinguished these on the basis of optimum salt concentration and temperature for growth, the production of acid from arabinose and sorbitol and the hydrolysis of gelatin. Sneath (1962) pointed out that Pohja's subgroups were chosen mainly on arbitrary characters and it appears to me that many of the characters chosen by Pohja from cluster analyses are largely unstable ones. Thus, the salt tolerance of acetoin-negative micrococci can be increased by subculture in media containing increasing concentrations of sodium chloride and may be decreased by subculture in laboratory media of normal salt concentration (Baird-Parker, unpublished). Similarly, optimum temperature for growth is largely dependent on growth conditions. Kocur & Martinec (1962) separated the halotolerant organisms belonging to my subgroups 5 and 6 into *Micrococcus varians* Migula, which forms acid from lactose and mannitol, and *M. conglomeratus* Migula

which does not attack either of these carbohydrates. However, in my experience, quite a number of the halotolerant glucose-oxidizing acetoin-negative micrococci oxidize lactose but not mannitol and vice versa. It must be concluded, therefore, that at present there is no satisfactory division of these organisms on physiological characters. It may be possible, however, to separate these organisms on their chemical compositions (Davison & Baddiley, 1963).

Micrococcus subgroups 7 and 8

Members of subgroups 7 and 8 either do not release detectable acid when carbohydrates are utilized or produce only small amounts of acids from these substrates. They also differ from most other micrococci in their inability to grow in the presence of 10% sodium chloride. The two subgroups contain a variety of morphological types, ranging from the irregular clusters of cocci characteristic of the strongly glucose-oxidizing micrococci to the tetrad and the cubical packet formers which were classified in the genus *Sarcina*. The two subgroups are distinguished by the red or pink pigmentation of cocci placed in *Micrococcus* subgroup 8.

The separation of subgroups 7 and 8 solely on the grounds of pigmentation is difficult to justify in the light of observations by several workers that *Micrococcus lysodeikticus*, which is a member of subgroup 7, is able to give stable pink-pigmented mutants (Field & Naylor, 1962; Dr G. W. Gould, personal communication). However, the cell-wall composition of the two subgroups is different (Table 5) as also are the proteins of their cell contents. It has also been shown by Eisenberg & Evans (1963) that two typical members of subgroup 7, *M. sodonensis* and *M. lysodeikticus*, have quite different carbon and nitrogen requirements from *M. roseus*. Therefore on this evidence it would appear justifiable to separate subgroup 7 from subgroup 8 on pigmentation.

The wide variety of morphological and physiological types placed in subgroup 7 is paralleled by the large number of species in which these organisms have been placed (Table 3). *Sarcina* spp. classifiable in *Micrococcus* subgroup 7 include: *S. aurantiaca*, *S. citrea*, *S. flava*, *S. lutea*, *S. marginata*, *S. variabilis*. The endospore-forming organism originally called *S. urea* (Beijerinck) Löhnis possesses similar physiological characters to the packet-forming micrococci but has been shown by MacDonald (1962) to be related serologically and in DNA composition to the Bacillaceae. It has therefore been re-classified as *Planosarcina ureae* Beijerinck by MacDonald (1962) and *Sporosarcina ureae* (Beijerinck) Orla-Jensen by Kocur & Martinec (1963*b*).

It is currently believed that intensive speciation of the weakly glucose-oxidizing micrococci is not justified and in recent classification they are placed in a single species. For example, Shaw *et al.* (1951) placed them in the species *Staphylococcus afermentans* ex Castellani and Kocur & Martinec (1962) in the species *Micrococcus luteus* (Schröeter) Cohn. This latter name would appear to be more appropriate as it excludes the implication that they are unable to utilize sugars; results obtained by Rosypal & Kocur (1963) by manometric techniques would indicate that they can all to some extent oxidize a variety of mono- and di-saccharides. Further, in detecting carbohydrate utilization by acid production, the medium used plays a large part in the result obtained (Cowan & Steel, 1964). Thus, Shaw *et al.* (1951) and Rosypal Kocur & Hoďák (1963) were unable to show acid production from glucose by *M.*

lysodeikticus growing in a peptone broth, although this organism will produce detectable acid when growing in a mineral-salt yeast-extract medium containing glucose.

Chemical composition of cells in relationship to classification

Several attempts have been made to use chemical components of micro-organisms for the classification of Gram-positive catalase-positive cocci Cummins & Harris (1956*a*, *b*) were able to divide nine named species of staphylococci, micrococci, sarcinas and aerococci into three groups according to differences in their cell-wall carbohydrates, amino acids and amino sugars. The first of these groups contained strains of *Staphylococcus aureus*, *S. albus*, *S. citreus*, *Micrococcus luteus* and *Sarcina lutea*; the second a strain of *Micrococcus conglomeratus* and strains of *Aerococcus viridans* and a third two red-pigmented organisms, *M. cinnabarius* and *M. rhodochrous*. However, as pointed out by Cummins & Harris (1956*b*) these groupings cut right across the divisions of these organisms on morphological and physiological grounds. Thus, the second group of Cummins & Harris is unlikely to be of taxonomic significance since it groups a typical micrococcoccus with strains of the physiologically very different *A. viridans*; their third group must also be disregarded as it contains two rod formers (Shaw *et al.* 1951; Cummins & Harris, 1956*a*). It would appear, therefore, that only the first group of Cummins & Harris is valid since it is the only one to contain only Gram-positive catalase-positive cocci. The characters of this group agree well with my results for a range of different physiological groups of staphylococci and micrococci (see Table 5) and indicate that qualitative differences in cell-wall sugars, amino sugars and amino acids are insufficient to distinguish well-defined groups of these organisms. Determination of these classes of compound is, however, of value in the separation of members of the Micrococcaceae from other coccus-forming genera. For example, I found, in agreement with the results of Salton & Pavlick (1960), that *M. varians* (NCTC 7281) contained diaminopimelic acid (DAP) in its cell wall in place of the lysine characteristic of other micrococci studied. The strains of *M. varians* was also atypical with respect to its morphology and physiological characters and therefore requires reclassification. Also, DAP was found in the cell walls of members of Abd-el-Malek & Gibson's group III B and *M. radiodurans* and *M. denitrificans*.

Qualitative differences in the composition of teichoic acids from the cell walls of staphylococci and micrococci were suggested by Davison & Baddiley (1963) to be of use in the classification of these organisms. With the exception of the type culture of *Staphylococcus saprophyticus* (NCTC 7292) ribitol-containing teichoic acids were found only in the cell walls of strains of *S. aureus*; teichoic acids were absent or present only as the glycerol polymer in the other coagulase-negative strains studied. The authors suggested that this ribitol-containing strain of *S. saprophyticus* (a member of *Micrococcus* subgroup 3) is atypical and found that a recently isolated 'pathogenic' strain of this species possessed only glycerol-teichoic acid. However, the occurrence of ribitol-teichoic acid may not be so uncommon amongst coagulase-negative staphylococci since Losnegard & Oeding (1963*a*) reported that two of four strains of *S. epidermidis* they examined contained ribitol-teichoic acids structurally and serologically related to the group A polysaccharide extractable from *S. aureus* (Losnegard & Oeding, 1963*b*). Perhaps it is significant that both these organisms

were isolated from urine. Davison & Baddiley's (1963) results clearly show the presence of at least two distinct glycerol-teichoic acids in organisms classifiable in my *Micrococcus* subgroup 5. Further studies of teichoic acids may therefore aid the classification of these micrococci and form the basis for serological studies of coagulase-negative staphylococci; see Davison *et al.* (1964).

Quantitative differences in the cell-wall amino acids of Gram-positive catalase-positive cocci were examined by Salton & Pavlik (1960), who found distinct differences in the molecular proportions of amino acids in the mucopeptides of staphylococci and micrococci; on a molecular basis the amount of glycine in the cell-wall mucopeptide of staphylococci was 4 times that present in the micrococci studied. The relative molecular proportions of lysine, glutamic acid, glycine and alanine were very similar for members of *Micrococcus* subgroup 7 (*Sarcina lutea*, *S. flava*, *M. tetragenus* and *M. lysodeikticus*); these amino acids were different both in type and proportion in *Micrococcus* subgroup 8 (*M. roseus*). Studies of C-terminal and N-terminal amino acids of the mucopeptide such as those mentioned by Salton (1960) may form an even finer taxonomic tool for classifying these organisms.

Gregory & Mabbit (1957), as part of a study of acetic acid-extractable amino acids and peptides in the classification of micro-organisms, examined representatives of cocci classified according to the scheme of Shaw *et al.* (1951). They found that strains of *Staphylococcus aureus* were distinguishable from *S. saprophyticus* by their pattern of ninhydrin-staining peptides and amino acids, but that a variety of patterns was produced by *S. lactis* strains, some of which were similar to *S. saprophyticus*; *S. roseus* and *S. afermentans* were also not clearly separated. My results are mainly in agreement with those of Gregory & Mabbit, but unlike them clear differentiation of strains corresponding to *S. aureus* and *S. saprophyticus* was not obtained and only two main groups could be distinguished (see Results). Two of the groups that Gregory & Mabbit distinguished chromatographically within *S. lactis* were Abd-el-Malek & Gibson's groups III A (*Micrococcus* subgroup 5) and III B. They found that the difference between chromatograms of these groups was greater than between the type strains of *S. aureus* and *S. saprophyticus*. This result would support my view that Abd-el-Malek & Gibson's group III B organisms are not members of the Micrococcaceae.

Electrophoretic separation of proteins from cell contents by starch-gel electrophoresis and staining of the proteins to demonstrate esterase activity was shown by Norris (1962) to be of use in the classification of *Bacillus* spp. Rosenkast & Clausen (1962) showed that the pattern of proteins separated by starch-gel electrophoresis of autolysed *Staphylococcus aureus* correlated with Cowan's three serological types. The present results indicate that the complex patterns of proteins separated electrophoretically from the cell contents of staphylococci and micrococci may be of value in the grouping of these organisms, but that further work along the lines used by Norris for *Bacillus* spp. may lead to more clear-cut results.

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REFERENCES

ABD-EL-MALEK, Y. & GIBSON, T. (1948). Studies in the bacteriology of milk. II. The staphylococci and micrococci of milk. *J. Dairy Res.* **15**, 249.

ANDERSON, J. I. W. (1962). Studies on micrococci isolated from the North Sea. *J. appl. Bact.* **25**, 362.

ARMSTRONG, J. J., BADDILEY, J., BUCHANAN, J. G., CARSS, B. & GREENBERG, G. R. (1958). Isolation and structure of ribitol phosphate derivatives (teichoic acids) from bacterial walls. *J. chem. Soc.* p. 4344.

BAIRD-PARKER, A. C. (1962). The occurrence and enumeration, according to a new classification, of micrococci and staphylococci in bacon and on human and pig skin. *J. appl. Bact.* **25**, 352.

BAIRD-PARKER, A. C. (1963). A classification of micrococci and staphylococci based on physiological and biochemical tests. *J. gen. Microbiol.* **30**, 409.

BARBER, M. & KUPER, S. W. A. (1951). Identification of *Staphylococcus pyogenes* by the phosphatase reaction. *J. Path. Bact.* **63**, 65.

BEAN, R. C. & PORTER, G. G. (1959). Detection and differentiation of sugars and polyols on single paper chromatograms. *Analyt. Chem.* **31**, 1929.

BROWN, R. L. & EVANS, J. B. (1963). Comparative physiology of antibiotic-resistant strains of *Staphylococcus aureus*. *J. Bact.* **85**, 1409.

CASTELLANI, A. (1955). Note préliminaire sur un nouveau microcoque isolé d'une dermatite axillaire superficielle tropicale. *Ann. Inst. Pasteur*, **89**, 475.

CLAUSEN, O. G. (1964). The discovery, isolation and classification of various α-haemolytic micrococci which resemble aerococci. *J. gen. Microbiol.* **35**, 1.

COLOMBO, D., CORBETTA, D., PIROTTA, A., RUFFINI, G. & SARTORI, A. (1960). A solvent for qualitative and quantitative determinations of sugars using paper chromatography. *J. Chromatog.* **3**, 343.

COWAN, S. T. & STEEL, K. J. (1961). Diagnostic tables for the common medical bacteria. *J. Hyg., Camb.* **59**, 357.

COWAN, S. T. & STEEL, K. J. (1964). Comparison of differentiating criteria for staphylococci and micrococci. *J. Bact.* **88**, 804.

CUMMINS, C. S. & HARRIS, H. (1956*a*). The chemical composition of the cell wall in some Gram-positive bacteria and its possible value as a taxonomic character. *J. gen. Microbiol.* **14**, 583.

CUMMINS, C. S. & HARRIS, H. (1956*b*). The relationships between certain members of the Staphylococcus-Micrococcus group as shown by their cell wall composition. *Int. Bull. bact. Nomencl. Taxon.* **6**, 111.

DAVIS, G. H. G. & BAIRD-PARKER, A. C. (1959). The classification of certain filamentous bacteria with respect to their chemical composition. *J. gen. Microbiol.* **21**, 612.

DAVIS, G. H. G. & FREER, J. H. (1960). Studies upon an oral aerobic actinomycete. *J. gen. Microbiol.* **23**, 163.

DAVISON, A. L. & BADDILEY, J. (1963). The distribution of teichoic acids in staphylococci. *J. gen. Microbiol.* **32**, 271.

DAVISON, A. L., BADDILEY, J., HAFSTAD, T., LOSNEGARD, N. & OEDING, P. (1964). Teichoic acids in the walls of staphylococci. *Nature, Lond.* **202**, 872.

DUTHIE, E. S. (1954). Evidence for two forms of staphylococcal coagulase. *J. gen. Microbiol.* **10**, 427.

EISENBERG, R. C. & EVANS, J. B. (1963). Energy and nitrogen requirements of *M. roseus*. *Canad. J. Microbiol.* **9**, 633.

EISLER, D. M. (1961). Coagulation of human plasma by *Pasteurella pestis*. *J. Bact.* **81**, 241.

ELEK, S. D. & LEVY, E. (1950). Distribution of haemolysins in pathogenic and non-pathogenic staphylococci. *J. Path. Bact.* **61**, 541.

ELLIOTT, S. D. (1962). Teichoic acid and the group antigen of Group D streptococci. *Nature, Lond.* **193**, 1105.

ELSON, L. A. & MORGAN, W. J. (1933). A colorimetric method for the determination of glucosamine and chondrosamine. *Biochem. J.* **27**, 1824.

EVANS, J. B., BRADFORD, W. L. & NIVEN, C. F., Jr. (1955). Comments concerning the taxonomy of the genera *Micrococcus* and *Staphylococcus*. *Int. Bull. bact. Nomencl. Taxon.* **5**, 61.

EVANS, J. B., BUETTNER, L. G. & NIVEN, C. F., Jr. (1952). Occurrence of streptococci that give a false positive coagulase test. *J. Bact.* **64**, 433.

FELTON, E. A., EVANS, J. B. & NIVEN, C. F., Jr. (1953). Production of catalase by pediococci. *J. Bact.* **65**, 481.

FIELD, A. K. & NAYLOR, H. B. (1962). Induction of lysogenic *Micrococcus lysodeikticus*. *J. Bact.* **84**, 1129.

FLETCHER, S. (1962). The growth of staphylococci in human serum. *J. Path. Bact.* **84**, 327.

FRÉDÉRICQ, P. (1941). Des actions coagulants et fibrinolytiques du staphylocoque, du bacille pyocyanique et de leurs coagulases. *Acta biol. belg.* **1**, 462.

FRÉDÉRICQ, P. (1946). Sur la coagulation du plasma oxalaté pas les cultures d'actinomycètes. *C. r. Seanc. Soc. Biol.* **140**, 1165.

GÜNTHER, H. L. & WHITE, H. R. (1961). The cultural and physiological characters of the pediococci. *J. gen. Microbiol.* **26**, 185.

GREGORY, M. & MABBITT, L. A. (1957). The differentiation of bacterial species by paper chromatography. V. Preliminary examination of the micrococci. *J. appl. Bact.* **20**, 226.

HEYWORTH, R., PERKINS, H. R. & WALKER, P. G. (1961). Paper chromatography of hexosamines and *N*-acetylhexosamines. *Nature, Lond.* **190**, 261.

HOARE, D. S. & WORK, E. (1957). The stereoisomers of α-diaminopimelic acid. II. Their distribution in the bacterial order Actinomycetates and in certain Eubacteriales. *Biochem. J.* **65**, 441.

HUGH, R. & LEIFSON, E. (1953). The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram-negative bacteria. *J. Bact.* **66**, 24.

ISAACS, A. & SCOULLER, J. M. (1948). Catalase production by Gram-positive cocci: a simple test for differentiating enterococcoci from micrococci. *J. Path. Bact.* **60**, 135.

JACOBS, S. I., WILLIS, A. T. & GOODBURN, G. M. (1963). Significance of deoxyribonuclease production by staphylococci. *Nature, Lond.* **200**, 709.

JENSEN, J. (1963). Apocatalase of catalase-negative staphylococci. *Science*, **141**, 45.

JONES, D., DEIBEL, R. H. & NIVEN, C. F., Jr. (1963). Identity of *Staphylococcus epidermidis*. *J. Bact.* **85**, 62.

KOCUR, M. & MARTINEC, T. (1962). *A Taxonomic Study of the Genus Micrococcoccus*. Brno (Czechoslovakia): Fac. Sci. Univ. Purkyně.

KOCUR, M. & MARTINEC, T. (1963*a*). The classification of some violet-pigmented micrococci. *J. gen. Microbiol.* **32**, 185.

KOCUR, M. & MARTINEC, T. (1963*b*). The taxonomic status of *Sporosarcina ureae* (Beijerinck) Orla-Jensen. *Int. Bull. bact. Nomencl. Taxon.* **13**, 201.

LANGSTON, C. W., GUTTIEREZ, J. & BOUMA, C. (1960). Catalase-producing strains of streptococci. *J. Bact.* **80**, 693.

LOMINSKI, I., SMITH, D. D. & MORRISON, R. B. (1953). A coagulase-destroying factor produced by variants of *Staphylococcus aureus*. *Nature, Lond.* **171**, 214.

LOSNEGARD, N. & OEDING, P. (1963*a*). Immunochemical studies on polysaccharides from *Staphylococcus epidermidis*. I. Isolation and chemical characterization. *Acta path. microbiol. scand.* **58**, 482.

LOSNEGARD, N. & OEDING, P. (1963*b*). Immunochemical studies on polysaccharides from *Staphylococcus epidermidis*: 2. Antigenic properties. *Acta path. microbiol. scand.* **58**, 493.

LUCAS, P. R. & SEELEY, H. W. (1955). A catalase negative *Micrococcus pyogenes* var. *aureus*. *J. Bact.* **69**, 231.

MACDONALD, R. E. (1962). Some further observations on the physiology and taxonomic position of *Planosarcina ureae*. *Bact. Proc.* p. G24.

MATTICK, A. T. R., CHEESEMAN, G. C., BERRIDGE, N. J. & BOTTAZZI, V. (1956). The differentiation of species of lactobaeilli and streptococci by means of paper partition chromatography. *J. appl. Bact.* **19**, 310.

MOSSEL, D. A. A. (1962). Attempt in classification of catalase-positive staphylococci and micrococci. *J. Bact.* **84**, 1140.

MUNCH-PETERSEN, E. (1961). Staphylococcal coagulase. *Zbl. Bakt.* (1 *Ref.*) **178**, 279.

NORRIS, J. R. (1962). Electrophoretic analysis of bacterial esterase systems—an aid to taxonomy. *J. gen. Microbiol.* **28**, vii.

PARKER, M. T. (1962). Phage typing and the epidemiology of *Staphylococcus aureus* infection. *J. appl. Bact.* **25**, 389.

PEREIRA, A. T. (1962). Coagulase-negative strains of staphylococcus possessing antigen 51 as agents of urinary infection. *J. clin. Path.* **15**, 252.

PLEVA, V. & KOCUR, M. (1963). Electron microscopy of the genera *Micrococcus* and *Staphylococcus*. *Reports from conference on taxonomy of bacteria held at J. E. Purkyně University, Brno, Czechoslovakia.* Brno: Pub. Fac. Sci. Univ. Purkyně No. 448.

POHJA, M. S. (1960). Micrococci in fermented meat products. Classification and description of 171 different strains. *Suom. Maataloust. Seur. Julk*, no. 93, 1.

POHJA, M. S. & GYLLENBERG, H. G. (1962). Numerical taxonomy of micrococci of fermented meat origin. *J. appl. Bact.* **25**, 341.

POULIK, M. D. (1957). Starch gel electrophoresis in a discontinuous system of buffers. *Nature, Lond.* **180**, 1477.

ROBINSON, J. & GIBBONS, N. E. (1952). The effects of salts on the growth of *Micrococcus halodenitrificans* n.sp. *Can. J. Bact.* **30**, 147.

ROSENKAST, P. & CLAUSEN, J. (1962). Classification of *Micrococcus* (*Staphylococcus aureus*) by means of electrophoretic mobility of extractable protein. *Experientia*, **18**, 293.

ROSYPAL, S. & KOCUR, M. (1963). The taxonomic significance of the oxidation of carbon compounds by different strains of *Micrococcus luteus*. *Antonie van Leeuwenhoek*, **29**, 313.

ROSYPAL, S., KOCUR, M. & HOŠÁK, K. (1963). A study of violet-pigmented micrococci. Yellow-pigmented mutants of *Staphylococcus flavocyaneus*. *J. gen. Microbiol.* **32**, 189.

ROY, J. K. (1960). A sensitive method for detecting sugars on paper chromatograms. *Analyst, Lond.* **85**, 295.

SALTON, M. R. J. (1959). An improved method for the detection of *N*-acetylamino sugars on paper chromatograms. *Biochim. biophys. Acta*, **34**, 308.

SALTON, M. R. J. (1960). *Microbial Cell Walls.* London: John Wiley and Sons, Inc.

SALTON, M. R. J. & HORNE, R. W. (1951). Studies of bacterial cell walls. II. Methods of preparation and some properties of cell walls. *Biochim. biophys. Acta*, **7**, 177.

SALTON, M. R. J. & PAVLIK, J. G. (1960). Studies of the bacterial cell wall. VI. Wall composition and sensitivity to lysozyme. *Biochim. biophys. Acta*, **39**, 398.

SCHINDLER, C. A. & SCHUHARDT, V. T. (1964). Lysostaphin: a new bacteriolytic agent for the staphylococcus. *Proc. natn. Acad. Sci., U.S.A.* **51**, 414.

SHAW, C., STITT, J. M. & COWAN, S. T. (1951). Staphylococci at their classification. *J. gen. Microbiol.* **5**, 1010.

SMITHIES, O. (1955). Zone electrophoresis in starch gels: Group variations in the serum proteins of normal human adults. *Biochem. J.* **61**, 629.

SMITHIES, W. R., GIBBONS, N. E. & BAYLEY, S. T. (1955). The chemical composition of the cell and cell wall of some halophilic bacteria. *Can. J. Microbiol.* **1**, 605.

SNEATH, P. H. A. (1960). A study of the bacterial genus *Chromobacterium*. *Iowa St. J. Sci.* **34**, 243.

SNEATH, P. H. A. (1962). The construction of taxonomic groups. In *Microbial Classification. Symp. Soc. gen. Microbiol.* **12**, 289.

SOLOMON, J. J. & SAN CLEMENTE, C. L. (1963). Some physiological characteristics of two sets of phage propagation strains of *Staphylococcus aureus*. *Appl. Microbiol.* **11**, 36.

SOMPOLINSKY, D. (1953). De l'impetigo contagiosa suis et du *Micrococcus hyicus* n.sp. *Schweiz. Arch. Tierheilk.* **95**, 302.

STEEL, K. J. (1964). *Micrococcus violagabriellae* Castellani. *J. gen. Microbiol.* **36**, 133.

SU, T. L. (1948). Micrococcin. An antibacterial substance formed by a strain of micrococcus. *Br. J. exp. Path.* **29**, 473.

TAKAHASHI, I. & GIBBONS, N. E. (1959). The effect of salt concentration on the morphology and chemical composition of *M. halo-denitrificans*. *Can. J. Microbiol.* **5**, 25.

THOMAS, E. T. & SCHUHARDT, V. T. (1964). Susceptibility of aerobic Micrococcaceae to lysostaphin. *Bact. Proc.* p. 23.

VERHOEVEN, W. (1957). In *Bergey's Manual of Determinative Bacteriology* (1957), 7th ed. Ed. by R. S. Breed, E. G. D. Murray & N. R. Smith, p. 457. London: Baillière, Tindall and Cox.

WHITE, F., RATTRAY, E. A. S. & DAVIDSON, D. J. (1963). Sensitivity to antibiotics and biochemical activities of serotypes of bovine staphylococci. *J. comp. Path.* **73**, 21.

WORK, E. (1964). Amino acids of walls of *Micrococcus radiodurans*. *Nature, Lond.* **201**, 1107.

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Thogoto Virus: a Hitherto Undescribed Agent Isolated from Ticks in Kenya

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SUMMARY

A filterable ether-sensitive agent was isolated from a pool of ticks collected during September 1960 from cattle in the Thogoto forest near Nairobi, Kenya. The pool consisted of *Boophilus decoloratus*, *Rhipicephalus appendiculatus*, *R. simus* and *R. evertsi*. The agent is apparently antigenically unrelated to 75 known arboviruses and is considered to be a previously undescribed virus, which has been named Thogoto virus. High degrees of immunity were found in livestock in some areas of East Africa.

INTRODUCTION

Ticks are known to be vectors of human and domestic animal virus infections in various parts of the world (Smith, 1962; Work, 1963). The tick-borne viruses which cause Russian spring–summer encephalitis and related encephalitic syndromes belong to a subgroup of Casals's serological Group B of the arboviruses (Casals & Brown, 1954). Two other Group B viruses are carried by Ixodid ticks: Powassan virus, which causes encephalitis in man in North America, and louping-ill virus, which produces a severe disease in sheep and occasional illness in cattle and man in Great Britain. On the other hand, Colorado tick fever virus and Kemerovo virus (Chumakov *et al.* 1963), which have been isolated from Ixodid ticks and which cause human disease in North America and the U.S.S.R. respectively, are not serologically related to Group B or to any other of the presently described arbovirus groups. The same applies to the previously known tick-borne viruses of Africa: Nairobi sheep disease, AR 492, Quaranfil, Chenuda and Nyamanini. These show by current techniques neither serological interrelationship nor cross-relationship with any other arbovirus. Nairobi sheep disease virus has been isolated from sheep, goats and *Rhipicephalus appendiculatus* during epizootics (Montgomery, 1917; Daubney & Hudson, 1931); AR 492 was isolated from *R. sanguineus* from the Sudan, Quaranfil and Chenuda from Argasid ticks in Egypt; Quaranfil has also been isolated from febrile children in Egypt, but no vertebrate involvement with either AR 492 or Chenuda virus has yet been demonstrated (Dr R. M. Taylor, personal communication). Nyamanini virus has been isolated from the cattle egret *Bubulcus ibis* (Linn.) and *Argas arboreus* collected in a heronry of cattle egrets and other birds in South Africa, and antibodies have been found in man, goats and a donkey

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(Dr B. M. McIntosh, personal communication). The present paper reports the isolation in Africa of another virus from Ixodid ticks, which is not serologically related to any other arbovirus so far tested, and to which high rates of antibody have been found in livestock in some parts of East Africa.

METHODS

Six steers were treated with insecticides and placed in the Thogoto forest in the Dagoretti-Ngong area on the outskirts of Nairobi, at an altitude of about 5600 ft. This forest was under the control of the Kenya forestry department and usually cattle were not permitted to enter the area.

Ticks. Ticks were collected daily by hand from the cattle into dry test tubes, during the latter half of 1960. They were delivered to the laboratory every 2 or 3 days. After identification they were pooled, usually by species but occasionally as a day's harvest. Each lot was then macerated with sand in a mortar and suspended in a balanced salt solution; about 1 ml. of solution was allowed for each tick. The supernatant fluid obtained by centrifugation at 2000 rev./min. for 15 min. was allowed to stand for 30 min. at room temperature; adult mice were then injected intraperitoneally (i.p.) with 0·1 ml. and infant mice intracerebrally (i.c.) with 0·01 ml. of the fluid.

Diluents. Hanks balanced salt solution containing 1000 i.u. penicillin, 1000 μg. streptomycin, 300 i.u. neomycin and 300 μg. mycostatin/ml., was used for initial suspension. Subsequent dilutions of animal tissues were made in 5% peptone water to which was added 500 i.u. penicillin and 500 μg. streptomycin/ml.

For the neutralization tests, the virus diluent was 0·75% bovine plasma albumin (Armour fraction V) in phosphate buffered saline (pH 7·4).

Mice. For isolation and early passage white mice from the Kabete colony were used. For identification studies, mice of the Entebbe colony were used; these are an albino Swiss strain derived from the stock of Carworth Farms, New York. Infant mice were inoculated at 2–3 days of age; adult mice when about 6 weeks old.

Hamsters. Golden Syrian hamsters were supplied from the Kabete laboratory colony soon after weaning. All injections (1 ml.) were made intraperitoneally.

Sheep. These were of mixed breed obtained from the Kabete laboratory farm or bought from outside sources.

Serological tests. Haemagglutination inhibition (HI) tests were done according to the methods of Clarke & Casals (1958). A haemagglutinating antigen was prepared from the livers of infected infant mice by sucrose acetone extraction, followed by fluorocarbon extraction to improve the cell pattern of the test. This antigen was used at 4° and pH 5·8. Several attempts to produce an antigen from mouse brain were unsuccessful.

Complement fixation (CF) tests used the same liver antigen as above, and followed the method of Weinbren (1958).

Protection tests (PT) were done in 1- to 3-day mice. These were inoculated intracerebrally with 0·02 ml. of mixtures of mouse-brain virus diluted to contain a calculated 100 LD50 and equal volumes of the undiluted test serum. These mixtures had been previously incubated at 37° for 1 hr.

RESULTS

Isolations

Infective agents were obtained from two lots of ticks collected during September 1960. Nothing was isolated from 25 pools of ticks collected later that year, comprising 16 pools of *Boophilus decoloratus* (Koch, 1844), 3 of *Ixodes* sp., 2 of *Rhipicephalus kochi* (Donitz, 1905), 1 of *R. appendiculatus* (Newmann, 1901) and 3 pools in which *R. evertsi* (Newmann, 1897), *R. simus* (Koch, 1844) and *R. appendiculatus* were mixed.

The first pool ('Thogoto 2A') from which a virus was isolated contained: 95 *B. decoloratus*, 14 *R. appendiculatus*, 14 *R. simus*, and 5 *R. evertsi*. These ticks were collected on 4 September 1960. Of twelve infant mice injected with a suspension of these ticks, seven were moribund on the 5th day while the remaining five were obviously sick; the adult mice showed no apparent reaction. Brains from two of the sick mice were diluted 1/100 and injected into other infant mice as well as adult mice and hamsters. The infant mice became sick on the 3rd and 4th days, the adult mice injected intracerebrally or intraperitoneally reacted sporadically from the 4th to the 8th day, while the hamsters were acutely sick on the 3rd day. The strain was passaged serially in groups of infant mice by the cerebral route. By the 15th infant mouse-brain passage, the virus titred 6·0 $\log_{10}$ LD 50/0·02 ml. by both the i.c. and i.p. routes, and deaths were occurring on the 3rd post-inoculation day.

Because of the uniformity of the reaction in hamsters passage was continued in this animal, with brain and spleen as inoculum. Severe illness with death followed in 2–3 days. A titration of an infected spleen was positive at a dilution of 10^{-6}, the highest dilution tested.

At autopsy it was observed that the brain, liver, and small intestine of these hamsters were markedly hyperaemic. Histopathological examination revealed no other changes in the viscera, but degeneration, particularly of the neurons, was observed in the cerebellum, where Purkinje cells were markedly affected. In addition, there was pronounced oedema with distension of the Virchow–Robin spaces. No perivascular cuffing was seen.

A 1% (w/v) suspension of hamster spleen was passed through a Ford S.B. Sterimat (R). Two hamsters injected with this filtrate reacted on the 3rd and 4th days whereas two which received unfiltered material died on the 3rd day.

A 1% (w/v) mouse-brain suspension in normal saline passed through Gradocol membranes of average pore diameter (apd) 680 and 410 mμ, but not through one of 210 mμ. These membranes were supplied by Dr F. Himmelweit of the Wright–Fleming Institute of Microbiology, St Mary's Hospital, London.

A 1% (w/v) hamster spleen suspension was exposed to ether by the method advocated by Andrewes & Horstmann (1949). Titration in hamsters showed untreated material to be infective at a dilution of at least 10^{-5}, but the treated material did not produce any apparent reaction.

Two sheep were injected intravenously with 5 ml. of 10% (v/v) brain suspension from the 5th infant mouse passage. Both sheep showed a thermal reaction on the 2nd and 3rd days, with temperatures up to 106° F. Blood taken at the height of the febrile reaction was injected into infant mice, hamsters and other sheep. In all these animals characteristic reactions were produced.

A pool ('Thogoto 3B') of 50 *Boophilus decoloratus* ticks, collected from the same cattle on 9 September 1960, was treated in the same way as the previous pool and injected into fourteen infant mice. On the 6th day three were sick, and on the 7th day one more, while the remainder appeared well on the 8th day when they were killed. The brain material was passed to other infant mice, which died sporadically from the 2nd to 6th days. From then on passages were continued in hamsters in which deaths occurred on the 2nd and 3rd days at each passage.

Identification

Strain Thogoto 2A was compared with 75 known arboviruses, 4 mouse viruses and 2 other viruses, each by one or more serological tests, with the results shown in Table 1.

Table 1. *Viruses used in the serological testing of Thogoto virus*

There was no reaction between Thogoto virus and any of the viruses listed below in haemagglutination inhibition tests or in complement fixation tests or in protection tests: in many cases more than one type of test was carried out.

Group A
- Aura
- Bebaru
- Chikungunya
- Eastern equine encephalitis
- Getah
- Mayaro
- Middelburg
- Ndumu
- O'nyong-nyong
- Semliki
- Sindbis
- Venezuelan equine encephalitis
- Western equine encephalitis

Group B (mosquito)
- Dengue 1
- Dengue 2
- Dengue 4
- H 336
- IPD A/249
- Japanese B encephalitis
- Murray valley encephalitis
- Ntaya
- St Louis encephalitis
- Spondweni
- Uganda S
- Usutu
- Wesselsbron
- West Nile
- Yellow fever
- Zika

Group B (tick)
- Powassan
- Russian spring–summer encephalitis (other)
- Entebbe Bat

Group C
- Apeu
- Caraparu
- Marituba
- Oriboca

Bunyamwera group
- Bunyamwera
- Germiston
- Ilesha

California complex
- California
- Lumbo
- Tahyna

Simbu group
- Akabane
- Ingwavuma
- Manzanilla
- Sathuperi
- Simbu

Bwamba group
- Bwamba
- Pongola

Bakau group
- Bakau
- Ketapah

Ungrouped
- African horse
- Bluetongue
- Koongol
- Lagos Bat
- Mossuril
- Nyando
- Rift Valley
- Sandfly fever (Naples)
- Sandfly fever (Sicily)
- Witwatersrand (tick)
- AR 492
- Chenuda
- Colorado tick
- Dalcairnie
- Dry Tortugas
- IG 619
- IG 690
- IG 700
- Kaisodi
- Kemerovo
- Nairobi sheep
- Nyamanini
- Quaranfil
- Silverwater

Other viruses
- Encephalomyocarditis
- Lymphocytic choriomeningitis recvirus (3?)*
- Semunya†
- Theiler's FA
- Theiler's GD 7

* Bell *et al.* (1964). † Weinbren *et al.* (1959).

Serological survey

Mouse protection tests were done with 48 human sera and 130 domestic animal sera; the latter were heat inactivated at 56° for 30 min. before testing. The human sera were from donors living in various parts of the Rift Valley in Kenya, between

Lakes Naivasha and Baringo, at altitudes ranging from 3000 to 8000 ft. above sea-level. None was protective against a challenge of 11 LD 50 doses. Some of the donors lived in close contact with livestock which included a proportion of immune animals (see below). Forty of these human sera were also screened against Thogoto haemagglutinating antigen, with negative results.

The domestic animal sera were collected in the same area of Kenya, with the addition of fifteen cattle sera from Entebbe, Uganda. The Nakuru cattle and sheep were pedigree exotic animals from farms at altitudes above 7000 ft. The Marigat livestock were local strains belonging to Samburu tribesmen, living at about 3000 ft. The Entebbe cattle were crossbreds living at 4000 ft.

The numbers of sera protective against a challenge of 60 LD 50 doses were as follows:

	Nakuru	Marigat	Entebbe
Cattle	0/33	12/20	15/15
Sheep	0/38	3/9	—
Goats	—	1/15	—

DISCUSSION

A virus (Thogoto 2A) has been isolated from a pool of ticks including *Boophilus decoloratus*, *Rhipicephalus appendiculatus*, *R. simus* and *R. evertsi* collected from cattle in the Thogoto forest near Nairobi, Kenya. A second isolate (Thogoto 3B) was made soon afterwards from a group of *B. decoloratus* obtained from the same locality. While this latter isolate resembled the first, it was not compared serologically with it. Because the isolation was made while work on the first was in progress, it is not possible to exclude an accidental laboratory cross-infection and it can therefore only be considered a possibility that *B. decoloratus* ticks are the actual carriers of infection.

The virus has been tested against all presently characterized African arboviruses, and other arboviruses whose distribution is not known to include Africa, in particular all presently known serologically ungrouped isolates from ticks. There was no haemagglutination-inhibition relationship with Russian spring–summer encephalitis antigen or any other of Casals's serological Group B, which effectively excludes that group of tick-borne agents. There was also no relationship with Nairobi sheep disease, the only other known African tick-borne virus disease of livestock. The only arboviruses against which the virus has not been tested are some isolates from the Americas and Australasia, and it seems unlikely that any of these will be found to be related to it; none was isolated from ticks. The results of the serological surveys do not indicate any involvement of man with the virus, but do suggest infection of livestock farmed at lower altitudes in Kenya and Uganda, where tick infestation is considerable.

We wish to thank the Director of Veterinary Services, Kenya, for permission to publish this paper, Mr M. L. Burdin for the histological examination, Mr P. Napier-Bax for collecting the ticks and Mr B. T. Parsons for their identification. Many of the HI and CF tests were done by Dr J. Casals (Rockefeller Foundation Virus Laboratories, New York). Some of these tests were done by one of us (J.P.W.) at

the Rockefeller Foundation Laboratories whilst in receipt of a Rockefeller Foundation travel grant. Dr M. C. Williams and Mr A. H. Revell of the East African Virus Research Institute collected the human and domestic animal survey sera.

REFERENCES

ANDREWES, C. H. & HORSTMANN, D. M. (1949). The susceptibility of viruses to ethyl ether. *J. gen. Microbiol.* **3**, 290.

BELL, T. M., MASSIE, A., ROSS, M. G. R. & WILLIAMS, M. C. (1964). Isolation of a reovirus from a case of Burkitt's lymphoma. *Brit. med. J.* i, 1212.

CASALS, J. & BROWN, L. V. (1954). Hemagglutination with arthropod-borne viruses. *J. exp. Med.* **99**, 429.

CHUMAKOV, M. P., SARMANOVA, E. S., BYCHKOVA, M. B., BANNOVA, G. G., PIVANOVA, G. P., KARPOVICH, L. G., IZOTOV, V. K. & RZHAKHOVA, O. E. (1963). *Vopr. Virusol.* **8**, 440, cited in *Biol. Abstr.* (1964), **45**, (7) 2385, 29542.

CLARKE, D. H. & CASALS, J. (1958). Techniques for hemagglutination-inhibition with arthropod-borne viruses. *Am. J. trop. Med. Hyg.* **7**, 561.

DAUBNEY, R. & HUDSON, J. R. (1931). Nairobi sheep disease. *Parasitology*, **23**, 507.

MONTGOMERY, E. (1917). On a tick-borne gastro-enteritis of sheep and goats occurring in British East Africa. *J. comp. Path.* **30**, 28.

SMITH, C. E. G. (1962). Ticks and viruses. *Symp. zool. Soc. Lond.* **6**, 199.

WEINBREN, M. P. (1958). An improved method for the complement fixation test using small quantities of reagents. *E. Afr. Virus Res. Inst. Rep.* 1957–58, 8, 38.

WEINBREN, M. P., KNIGHT, E. M., ELLICE, J. M. & HEWITT, L. E. (1959). Hitherto unknown strains of virus isolated from human serum. *E. Afr. Virus Res. Inst. Rep.* 1958–59, **9**, 7.

WORK, T. H. (1963). Tick-borne viruses. *Bull. Wld Hlth Org.* **29**, 59.

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The Structure of the Head, Collar and Base-Plate of 'T-even' Type Bacteriophages

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SUMMARY

A major feature in the classical conception of the morphology of the T-even type coliphage is the shape of the head, believed to be a bipyramidal hexagonal prism. The present paper shows that this shape is not consistent with the appearance in electron micrographs, and an alternative model is proposed. The fine structure of the head is compared with that of polymerized head protein (polyhead) obtained from naturally-occurring lethal mutants. Certain hexagonal and star-shaped objects have been described in the literature as base-plates. Because of inconsistencies in their appearance, they have now been measured and studied. The results given here indicate that the star-shaped objects are disorganized base-plates and the hexagonal objects are collars. By using the rotation method for printing micrographs of collars it is found that they have a hollow subunit at each corner.

INTRODUCTION

Despite the fact that coliphages of the T-even morphological type have been studied more than any other kind, several questions about their molecular structure remain to be answered. This is mainly because they are complex bodies with numerous structural components, whose functions are in many cases uncertain. This paper seeks to add further details to the picture which has been built up in the past.

Investigations hitherto have shown that the T-even type phage consists of a head, believed to be in the form of a bipyramidal hexagonal prism (Williams & Fraser, 1953) and a complicated contractile tail, which has been the subject of the most intensive study. The tip of the tail consists of a hexagonal base-plate with a pin attached to each corner. A thin hollow tube or core is firmly attached to the base plate and runs up the centre of the tail to the head of the phage where it is fixed to the apex of one of the pyramids of the bipyramidal hexagonal prism. A sheath which forms the contractile part of the tail surrounds the core and extends almost to the head (Brenner *et al.* 1959). In the normal or extended state, i.e. before nucleic acid injection, it consists of some 144 morphological subunits related by screw symmetry and arranged in a series of 24 annuli of sixfold radial symmetry (Bradley, 1963). It is believed that the sheath contraction which accompanies nucleic acid injection is brought about by doubling the number of subunits per annulus (from six to twelve), a hypothesis which is supported by observations with the electron microscope. Between the top of the sheath and the head there is a disc-like collar (Anderson, 1960; Bradley, 1963) which almost certainty has a sixfold radial symmetry. Several fibres, which correspond to the tail fibres seen by Brenner *et al.*

(1959), are attached to both collar and base-plate, and form a network round the sheath. These fibres are closely associated with the adsorption process; under conditions when the phage can adsorb and infect, the network breaks at the collar, and the fibres attached only at the base-plate can wave about at random in the medium. As soon as they touch the host bacterium the base-plate is brought into intimate contact with the cell wall. If the phage is placed in a medium in which adsorption cannot take place (e.g. in the absence of suitable cofactors such as tryptophan for coliphage T 4) the fibres are found as the network round the sheath (Professor E. Kellenberger and colleagues, personal communication). The phage head contains double-stranded deoxyribonucleic acid (DNA) which can be unravelled and seen in the electron microscope as a single filament 49 μ long (Kleinschmidt, Lang, Jacherts & Zahn, 1962). One free end of this fibre extends down the inside of the core to the base-plate.

The structures of a few of these features have yet to be fully described or clarified. The most important is the phage head; the number, size and arrangement of the morphological subunits or capsomeres are still unknown. Also, there are certain features in electron micrographs of the head which strongly suggest that the classical model for its geometrical form is incorrect or at least that the head is pleomorphic. In the tail, the only features which are not clearly described are the collar and the detailed structure of the base-plate. These points are discussed here on the basis of electron micrographs.

Preparations of single phage components for electron microscopy may be obtained either by chemical and physical separation (Brenner *et al.* 1959; Anderson & Stephens, 1964) or from lysates of lethal mutations of the phage. Artificially-induced lethal mutants of coliphage T 4 were studied by Epstein *et al.* (1964), Favre *et al.* (1964) and Eiserling & Boy de la Tour, 1964). They showed amongst other things that in such cases only one phage component or component protein may be produced in the host bacterium. Some mutants caused the host cell to produce tail cores with base-plates attached, while others induced the formation of sheath or head protein. The latter were called polysheath and polyhead, respectively. Since both are chemically and serologically identical with the true phage components, one might expect a structural relationship to exist. These molecular assemblies are easy to identify in electron micrographs of lysates or sections of infected bacteria and are described here in association with new isolates of T-even phages, indicating that such mutants can occur naturally.

METHODS

Sources of coliphages. Coliphage BP/4 was isolated from the River Ayr at Ayr; phage PVL was obtained from a manure heap near Biggar, Lanarkshire; and phage WB/5 from a canal in Vienna. Coliphage T 4 was supplied by Dr and Mrs K. G. Lark (Kansas State University).

Isolation, growth and purification of bacteriophages. The methods used were the same as those described previously, with *Escherichia coli* strain c 2 as a host organism (Bradley, 1963). Suspensions for electron microscopy were made by extracting confluently lysed plates with neutral 0·1 M-ammonium acetate. Purification was by several cycles of centrifugation.

Treatment of coliphage T4 with antiserum. Anti-T 4 serum was kindly supplied by Dr J. G. Howard (Edinburgh University). A phage suspension in broth was decreased to about 25 % of its original activity by adding a suitable dilution of antiserum and incubating at 37° for 15 min. The phage particles were then centrifuged at 15,000*g* for 1 hr and resuspended in 0·1 M-ammonium acetate (neutral) for electron microscopy.

Electron microscope specimen preparation. The negative contrast method was used; most of the embedding mixtures have been mentioned elsewhere (Bradley, 1962). Solutions of uranyl salts containing ethylenediaminetetra-acetic acid (EDTA) were most suitable for the preservation of the shape of the phage head. Uranyl formate (Finch, 1964) required a special procedure for the preparation of a 2 % solution, the reason being that this compound is not easily obtainable from the major chemical manufacturers. It is best prepared in the laboratory as follows. Uranyl nitrate (cryst.; 2·79 g.) is dissolved in about 30 ml. water and ammonium hydroxide added to excess. The resulting canary yellow ppt. of uranyl hydroxide is centrifuged at 2000*g* for 2 min. and the colourless supernatant fluid discarded. The ppt. is washed free from ammonia by centrifugation and finally resuspended in about 30 ml. water. The addition of 0·5 ml. of 90 % formic acid will just dissolve the uranyl hydroxide, providing a solution of uranyl formate, which on making up to 100 ml. will contain 2 % (w/v). This solution can be applied to grids in the normal way, but it is not stable, a ppt. forming within a few days. It is therefore best to make a fresh solution when required.

Printing electron micrographs. To obtain greater detail from some of the electron micrographs obtained, the rotation method was used for printing them (Markham, Frey & Hills, 1963). Suitable areas were first selected by rotating normal prints at several hundred rev./min. under stroboscopic illumination. The negatives were then placed in the enlarger, the selected areas centred over a gramophone turntable, and the printing paper placed on the turntable. Since the specimens concerned obviously possessed a sixfold rotational symmetry, prints were made by using six exposures, the turntable being rotated one sixth of a revolution between each. Particular care is required in the interpretation of such prints.

RESULTS

The shape of the normal phage head

Electron micrographs of the head showed features which were inconsistent with the classical model of a bipyramidal hexagonal prism. These consisted of rhomboids and other shapes on the faces of the main body of the head, supposedly a right hexagonal prism; typical examples are shown in Pl. 1, fig. 1. More or less similar shapes can be found in some published micrographs (e.g. Brenner *et al.* 1959, Pl. IV; Cummings & Kozloff, 1962; Anderson & Stephens, 1964, fig. 4). In Pl. 1, fig. 2, the supposed prism has a triangular face on it. On the other hand, profiles of phage particles where the support film has folded (Pl. 1, fig. 3) would appear to be those of the classical figure; therefore any proposed new shape would have to be close to a bipyramidal hexagonal prism and difficult to distinguish from it. Such a figure can be deduced from electron micrographs and can be obtained by rotating one pyramid by 30° with respect to the other and replacing the prism by a series of triangles as

shown in the model in Pl. 1, fig. 4. The slight departure from the vertical of the outer edges of the main body of Pl. 1, fig. 4 (left) is due to tilt in the copying camera; this would not show with the virtually parallel illumination of the electron microscope.

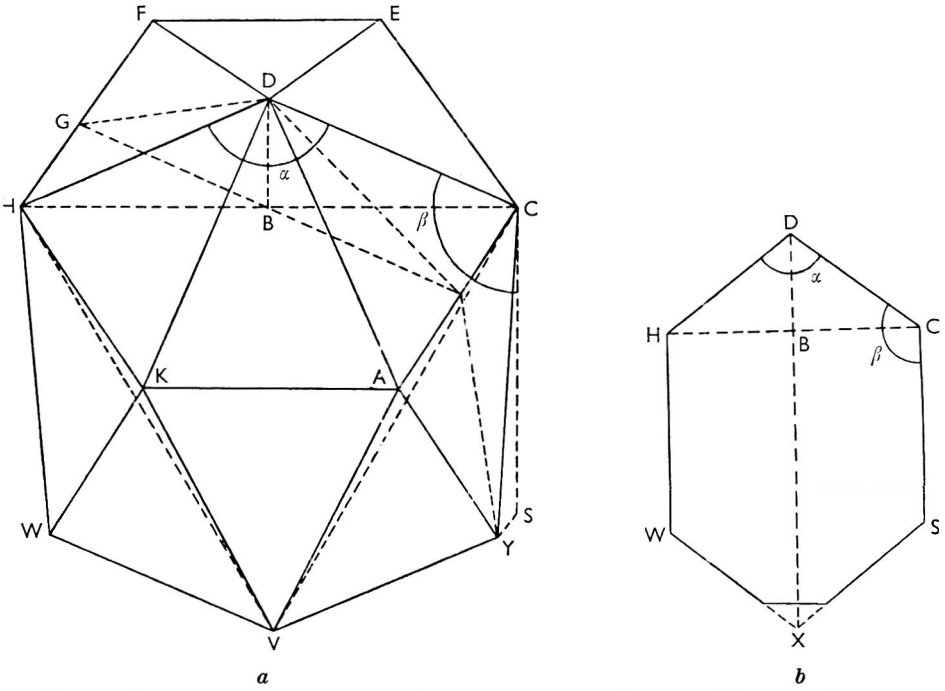

Fig. 1. (*a*) Diagram of part of proposed figure for T2 type phage heads; CS is perpendicular to the plane of the hexagon EFHKAC; it represents the projection of an edge (e.g. CY) when the whole figure is rotated in either direction by 30° to correspond to Pl. 1, fig. 4 (left). (*b*) Diagram of outline of phage head as seen in electron micrographs.

In the heads in Pl. 1, figs. 5, 6 several faces are visible; by no stretch of the imagination can they be attributed to a hexagonal prism, but the superimposed wire models of the proposed figure are much closer. Some of the edges in the models have been erased and replaced by broken lines to accentuate the sides corresponding to those visible in the micrographs. One frequently finds the top of the phage head wider than the bottom, or vice versa (Pl. 1, fig. 7; see also Brenner *et al.* 1959, Pl. III), and again the wire model shows how this can come about, though the pyramids of the model are more acute than those of the phage, probably due to distortion. The reliability and implications of these observations are discussed below.

The dimensions of the normal phage head

To prepare a model of a normal phage head and to consider its structure the various dimensions must be ascertained. This has been achieved by taking measurements from micrographs of phages in various negative-staining media and averaging the results. The measurements are given in full in Table 1, the various sides and angles referring to Fig. 1, which is drawn from the model described above.

To test further the validity of the model, the acute angles which are indicated by arrows in Pl. 1, fig. 1, were measured on a number of micrographs; these corresponded to the angles AVY or KWV in Fig. 1. The results are given in Table 2, and their significance is discussed below. It was found that the best negative-staining media for the three-dimensional preservation of large objects were uranyl acetate with EDTA and a mixture of sodium molybdate and ammonium carbonate (Bradley, 1962). On the whole, T 2 type heads tend to collapse in phosphotungstate (PTA) so that the majority of heads whose measurements are given in Table 1 and 2 were embedded in the uranyl or molybdate media.

Table 1. *Dimensions of T2 type phage heads (see Fig. 1)*

Phage type	Negative stain	α	β'	DC	DX	CS	DB	HC
T4	Na molybdate	117	121	410	1000	480	260	710
T4	Na molybdate	107	126	430	1100	570	263	710
$\phi 66t-$	PTA	96	132	560	1350	600	375	825
$\alpha 1$	PTA	108	131	530	1350	660	345	810
T2	Uranyl nitrate	107	125	490	1150	550	300	725
T2	Uranyl nitrate	109	125	460	1100	550	275	725
T2	Uranyl nitrate + EDTA	103	128	530	1250	580	335	860
T2	Uranyl nitrate + EDTA	117	121	490	1200	670	265	800
T2	Uranyl nitrate + EDTA	110	124	520	1300	710	295	815
T2	Uranyl nitrate + EDTA	115	122	510	1200	650	275	850
T2	Uranyl acetate	96	131	520	1450	700	375	850
T2	Uranyl acetate	118	122	500	1300	780	260	800
T2	Uranyl acetate + EDTA	99	132	600	1450	650	400	880
T2	Uranyl acetate + EDTA	102	129	550	1400	700	350	900
T2	Uranyl nitrate shadowed	105	128	500	1250	630	310	825
T2	Uranyl nitrate shadowed	106	129	540	1350	600	375	850
	Averages	107°	127°	510 Å	1260 Å	630 Å	315 Å	810 Å

Note: α is average of angles at D and X; β is average of angles at H, W, C and S; DC is average of DC, DH, WX and XS; CS is average of CS and WH; DB is calculated from DB = $\frac{1}{2}$(DX − CS); HC is average of HC and WS.

Table 2. *Values of acute angle indicated in Pl. 1, fig. 1*

Phage type	Negative stain	Number and origin of micrographs	Angle°
T2	Uranyl acetate + EDTA	7 measurements, Bradley, unpublished	68·5 ± 4·5*
T4	Sodium molybdate + ammonium carbonate	7 measurements, Bradley, unpublished	69 ± 4*
T2	PTA	1 measurement, Brenner *et al.* (1959) Pl. IV	78
T2	PTA	1 measurement, Fernandez-Moran (1962), fig. 3	70
T2	Potassium silicotungstate	1 measurement, Anderson & Stephens (1964), fig. 1	78
T2	PTA	1 measurement, Bradley, unpublished	74
		Average of PTA stained particles	75

* Maximum deviation.

The structure of normal phage heads

No properly organized capsomere structure can be seen in micrographs of either full or empty phage heads, regardless of the negative-staining medium used. This is in direct contrast to the appearance of polyhead which is described below. In an attempt to reveal some form of ultrastructure the negative stain uranyl formate which had given so much success with tobacco mosaic virus (Finch, 1964) was used. The results (Pl. 2, fig. 8) showed a very fine periodicity (arrowed). In Pl. 2, fig. 9, the lines ran parallel to the edges of the head, producing a whorled effect. A very close examination revealed circular objects which might be disorganized capsomeres.

Empty heads revealed only the familiar ghost appearance; two are included here embedded in sodium molybdate and ammonium carbonate (Pl. 2, fig. 10) to show a small lump of protein within the head at the point of attachment of the tail core. In these two particles the DNA was lost, without sheath contraction.

Plate 2, fig. 11, was obtained in an attempt to mark the sites of individual capsomeres by adsorbing anti-T 4 antibodies to the phage head. A number of short rods can be seen at the top of the micrograph. Their centre to centre spacing is about 70 Å, but the diameter of the visible part of the rod (the white portion) is about 50 Å. Such rods were frequently found on other micrographs of partly inactivated phage preparations. Their significance will be discussed later.

The structure of polyhead

The general appearance of the polyhead at low magnification is shown in Pl. 2, fig. 12; an intact phage particle and a short length of polysheath are included in the field for comparison. The polyhead is more or less uniform in width save for a few places where it narrows and becomes penetrated or covered by the PTA negative staining medium. A part of this micrograph is enlarged in Pl. 2, fig. 13; it can be seen that there is a mottling on the polyhead which suggests a subunit structure; this is not present on the intact phage head. In cases where the polyhead is well preserved the, arrangement of these subunits can be seen (Pl. 3, fig. 14). It appears that they are packed in a regular hexagonal array, and this can be confirmed by using the rotation method for printing the micrograph; the area marked in Pl. 3, fig. 14, has been treated in this way and is shown in Pl. 3, fig. 15. Here the subunits are well defined and show a remarkably regular packing over a large area. Plate 3, fig. 16 strongly suggests that they have a regular hexagonal outline (arrows), a shape which is essential to produce the observed packing. Their appearance also indicates that the PTA penetrated them in a direction perpendicular to the plane of the polyhead surface, suggesting a tubular form. A glance at the right-hand edge of the polyhead in Pl. 3, fig. 14, where the subunits are more or less in profile, confirms this.

The widths of the cylindrical portions of pieces of polyhead were found to vary from 870 ± 30 Å to 1000 ± 30 Å. This indicates that the number of subunits round the polyhead was also variable. The widths of the narrowest parts of the polyhead (e.g. Pl. 2, fig. 13) were 660 ± 30 Å. The average centre-centre spacing of the subunits was 72 Å (23 measurements) from normal prints and 70·5 Å (30 measure-measurements) from Pl. 3, fig. 15. The spacing of the lines of subunits down the long axis of the polyhead (lateral striations on Pl. 3, fig. 14) was 60 Å (20 striations were

1200 Å). From Pl. 3, fig. 15, the result was also 60 Å. One may use this figure to calculate the centre-centre spacing of the subunits to cheek the direct measurements given above (60 Å sec. 30°) the result being 69·3 Å. It is considered that this figure and the value of 70·5 Å obtained from the rotated prints (Pl. 3, fig. 15) are more accurate than the 72 Å value taken from normal prints, because of the difficulty in measuring the latter properly. The average of the former two measurements is virtually 70 Å; this will be taken as the final value.

The diameter of the tail core

A component which might well correspond in diameter to an individual head capsomere is the tail core. This was carefully measured from micrographs of preparations of lethal mutations which produce cores with base-plates attached (Pl. 3, fig. 17). This core is far too long to fit a normal phage particle, but its diameter is 70 Å, which agrees with the value found for cores by other authors.

The dimensions of collars and base-plates

To assist in differentiating between collars and base-plates found detached from virions, the diameters of various star-shaped or hexagonal discs figured by other authors and in the present paper were measured. The results are given in Table 3. Objects 1–3 are hexagonal discs with apical subunits and are considered to be collars. Objects 6–8 are star-shaped and are disrupted base-plates.

Table 3. *Dimensions of collars and base-plates*

Object	Max. Å	Average Å	Min. Å
1. Disc, Brenner & Horne (1959), fig. 6	400	375	350
2. Disc, Anderson & Stephens (1964), fig. 7–9	410	360	310
3. Disc, present paper, Pl. 4, fig. 23	430	375	320
4. Collars on intact phage, 8 measurements, Bradley, unpublished	—	360	—
5. Base-plates on intact phage, 8 measurements, Bradley, unpublished	—	305	—
6. Star, Fernandez-Moran (1962), fig. 2	600	—	—
7. Star, Anderson & Stephens (1964), figs. 4, 5	600	—	—
8. Star, present paper, Pl. 3, fig. 20	580	—	—

Note: maximum values were obtained by measuring the diameter from apex to apex and minimum values by measuring from the centres of opposite sides. On intact phages where the orientation was unknown, the value obtained was considered as an average.

The base-plate and tail pins

In the normal way, the phage base-plate and tail pins are seen in profile (Pl. 2, fig. 10). In this micrograph one can also see two small pins pointing upwards towards the head. These are clearer in Pl. 3, fig. 18, where they are surmounted by a pyramid-like structure, which has obviously been revealed by the penetration of the negative stain into the sheath, for it is clearly an internal structure. With most negative-staining media, the base-plate and tail pins have a tendency to break up into what is usually a mass of fibres, but which on occasion takes the form of a star, shown partly bent over in Pl. 3, figs. 19, 20. It is to be noted that the tail fibres

have remained attached to it. Anderson & Stephens (1964) showed a particularly good micrograph of this structure; it is reproduced here in Pl. 4, fig. 21.

The collar

As with the base-plate the collar is normally seen in profile (Pl. 4, fig. 22) fitting closely to the flattened apex of the basal pyramid of the head. When detached from the virion, the collar is a six-sided disc with a morphological subunit at each apex and a central plug of protein (Pl. 4, fig. 23). A much clearer picture can be obtained by using the rotation method for printing the plate (Pl. 4, fig. 24). A careful comparison of the two micrographs is required for correct interpretation; only features which show a marked increase in contrast in the rotated print as opposed to the original, can be considered real. Thus, the hollow apical subunits are a true representation, and probably the central plug, but the ring of relatively indistinct subunits in between is an artifact. It is to be noted that there were no tail fibres attached to the collar.

Pl. 4, fig. 23, is very similar to several micrographs of objects described by Anderson & Stephens (1964) as base-plates; it will be shown below that these are, in fact, collars.

DISCUSSION

The shape of the T 2 phage head. The heads in Pl. 1 fig. 1, show diagonal lines and faces in the centre portions which are clearly not hexagonal prisms. The most obvious argument against such evidence is that the heads have become distorted. However, this would be unlikely to produce straight lines, indeed a familiar pattern indicating distortion and collapse consists of an irregular band across the centre of the head (see Bradley, 1963, Pl. 1, fig. 5); also the clarity of the faces in Pl. 1, fig. 5, is not consistent with this criticism. The reason why this observation has been overlooked in the past is that most of the electron microscopy done with T 2 type phages has involved negative staining in PTA. I showed (Bradley, 1962) that the heads collapse very easily in this medium so that clear facets will be seen only occasionally, though diagonal lines, etc., are more frequent, as mentioned above.

The present model was arrived at by trial and error and is more a working hypothesis than a definite proposal. Its accuracy may be judged by comparing the wire silhouettes with the heads; the various edges showing in the micrographs correspond closely with those on the model. Also, the angle made by the diagonal lines with the short axes of the head (Table 2), may be measured and compared with that of the model. It can be seen that such an angle would correspond to the basal angles of the isosceles triangles making up the centre portion of the head. This angle can be calculated from the appropriate average measurements (given in Table 1) or measured directly from the model (Pl. 1, fig. 4) which has been constructed according to these figures. In either case the result is 72°, close to the value given in Table 2.

The rotation of the two pyramids with respect to one another can, of course, produce shapes in the intermediate section which are different from that postulated. One alternative is the joining of the two other points of the rhomboids (HV and VC in Fig. 1) to form two obtuse-angled triangles bending inwards slightly. Secondly, there need not necessarily be any triangles at all, the rhomboid surfaces being warped to accommodate the curvature. These arrangements would produce more

strain in the capsid, and since they are not consistent with the evidence of the electron micrographs, are considered to be improbable.

The average dimensions in Table 1 correlate accurately with one another. For example, the angles α and β were measured independently and are not related on electron micrographs, but it can be seen from Fig. 1 that in triangle DBC, $\frac{1}{2}\alpha+\beta-90°$ should equal 90°. When calculated from α and β in Table 1, the result is $90\frac{1}{2}°$. The same degree of accuracy holds for other unrelated measurements. Indeed, if this were not so, it would not be possible to construct a model with all the dimensions correct. However, the observations in Table 1 are applicable to a bipyramidal hexagonal prism as well as to the proposed figure and cannot be used as evidence in favour of the latter.

One should note that the angles α and β and the length HC will vary slightly according to the orientation of the pyramids. The alternatives are shown in Fig. 1 as the angles GDI and DIY and the length GI. If the model is a true one, the dimensions measured will be an average of those obtained with each orientation, since values from both pyramids were taken into account. In any case, the differences are small and outside the limit of accuracy of measurement, particularly when different negative stains are used.

This new shape will in no way change the conception of the virus as a whole. The axis to which the tail is attached still has a sixfold radial symmetry, and the head volume will be unchanged. However, one definite and important possibility must be considered, namely that the head may be pleomorphic. Cummings & Kozloff (1960, 1962) claimed that the T 2 phage can have two different head lengths according to the environment. The 15% difference is located in what was originally the prismatic portion, the two pyramids remaining unchanged. They did not postulate any change in the shape of the head, but on the basis of the present model it is easy to see how its length could be changed: any rotation of the pyramids with respect to one another could alter their distance apart. In addition, such a rotation could change the geometrical figure from a bipyramidal hexagonal prism to the proposed new one, or vice versa. Under these circumstances, therefore, there would be no reason why both head forms should not exist.

The fine structure of normal phage head and polyhead. Having re-established the shape of the head with some degree of certainty, the next step is to try and relate any capsomere structure with it. Unfortunately micrographs such as Pl. 2, figs. 8, 9 do not show a regular structure, and great care must be exercised in interpreting such detail as can be discerned, since its magnitude is near that of the background structure of the negative stain. Here the instrumental resolution is considerably better than 10 Å, so that the negative-stain background, which is of this order, is quite clear. This will be superimposed on any structure on the phage (magnitude about 20 Å), rendering it less clear. The best approach is to compare the background with the head structure. The first most noticeable difference is the whorled appearance shown particularly clearly in Pl. 2, fig. 9. This was visible to some extent on most heads embedded in uranyl formate, and may thus be considered real. The spacing of these concentric lines is 20 Å. They can only be due to capsomeres or the nucleic acid within the capsid. The latter is not as far-fetched as may at first appear since uranyl ions will penetrate the capsid and combine with the DNA, effectively staining it (Bradley, 1962). The spacing is correct and indeed one might expect the

nucleic acid strand to be wound up in this orderly fashion. The argument against this is that the whorls are definitely beaded, suggesting 20 Å capsomeres. In Pl. 2, fig. 8, possible capsomeres are more obvious, but the whorls are indistinct. It must be remembered, however, that a beaded appearance can be produced by local aggregation of a stain like a shadowing material. This would give an appearance resembling the shadowed nucleic acid strands of Kleinschmidt *et al.* (1962). On the whole, it is considered that the whorled lines represent the nucleic acid since capsomeres would show an ordered array which is not present in these micrographs.

The attempt at marking the individual capsomeres with antibody (Pl. 2, fig. 11) was made on the assumption that if each antibody molecule attached itself to one capsomere, their arrangement might thus be revealed. As it happened, the antibody molecules were large, and there were too few present, but it is notable that their spacing on the phage head was the same as that of the polyhead subunits.

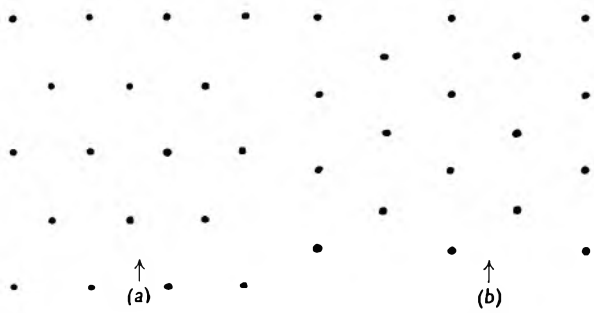

Fig. 2. Diagram of the hexagonal array of subunits in phage PVL polyhead; the arrows show the long axis of the polyhead tube; (*a*) normal orientation, (*b*) unusual orientation as figured by Eiserling & Boy de la Tour (1964).

One other object must be noted: the lump of protein within the head membrane (Pl. 2, fig. 10). This is doubtless an array of molecules firmly attaching the bulky tail in a manner analogous to that of phage ZG/3A (Bradley, 1964).

At this point the structure of polyhead must be considered to see whether there is a relationship between it and the normal head structure. The array of subunits in polyhead is subject to variations in number and arrangement. The varying width of the separate pieces of polyhead (800–1000 Å) shows that the number of subunits round the circumference is not constant. Also the orientation of the hexagonal array with respect to the long axis sometimes differs by 90°; the different arrangements are drawn in Fig. 2. Fig. 2*b* is illustrated by Eiserling & Boy de la Tour (1964) and is very unusual. Nevertheless, we are led to the inescapable conclusion that polyhead is nothing more than an assembly of head protein subunits, arranged in the most convenient packing for a cylindrical shape rather than a body with distinct facets. The most important question which arises is, why are the polyhead subunits not visible on the normal head? The most attractive answer is that the polyhead morphological subunits are composed of the same chemical subunits as the phage head proper, but that they in fact represent larger aggregates which are themselves arranged in a hexagonal packing. Such chemical units could only be

20 Å or so in size and difficult to resolve on the phage head; they would, nevertheless, possess the same chemical and serological characteristics. On the other hand it seems almost too much of a coincidence that the diameter of the tail core and the antibody molecules in Pl. 2, fig. 11, are both about 70 Å, the same size as the polyhead subunits. In short, it is impossible to postulate any definite structural relationship between polyhead and the normal phage head.

The collar and base-plate. These two components have several features in common: both are hexagonal discs, are attached to the tail core and have apical subunits. It is therefore not altogether surprising that they can easily be confused when found as isolated units. It is obviously necessary to establish distinguishing characteristics unequivocally and to ensure that objects in micrographs such as that shown in Pl. 4, fig. 23, are in fact collars.

A most valuable micrograph by Anderson & Stephens (1964, Pl. 4, fig. 21), shows clearly that the star-shaped object has become detached from the end of the core and is thus, without question, a base-plate. This is confirmed by Pl. 3, figs. 19, 20, which show the star still attached to the base of the tail sheath. It must now be established whether the object seen in Pl. 4, fig. 23, is a collar or a base-plate in some intermediate stage of deformation, as suggested by Anderson & Stephens (1964). Let us consider the morphological differences and their implications. First, it will be found that published electron micrographs show the star with all or most of the tail fibres attached (e.g. Fernandez-Moran, 1962) as in Pl. 4, fig. 21, whereas the suspected collars usually have no fibres at all, and very rarely one or two. Now the bond between the tail fibres and the base-plate is strong because electron micrographs show the majority of them still attached even after the base-plate has started to become disorganized. On the other hand, fibres are attached to both base-plate and collar in the intact phage only under specific conditions (see Introduction), but it is known that the bond between fibres and collar is reversible. Finally, one never finds fibres attached to the collar and not the base-plate. We may thus say that hexagonal discs with fibres attached are base-plates and objects without fibres attached are collars.

A second difference is that the star-shaped base-plates have a hole in the centre whereas the supposed collars have a plug of protein. It is clear that the hole has been caused by the base-plate having been torn from the core (as in Pl. 4, fig. 21). As with the tail fibres, the bond between the base-plate and core is strong, since these two components are usually found joined together in lysates but seldom apart. The base-plate-to-core bond only weakens under conditions of sheath contraction when the base-plate slides up the core and remains attached to the sheath; it is not released into a free state. It is thus very unlikely that base-plates will be found in a free state without a hole; this would involve snapping-off a short length of core, an improbable state of affairs. A different relationship exists between the collar and the core. The collar is attached at the weakest point on the tail, namely, where the 70 Å core joins the massive tail assembly to the head. The strain here will be considerable and the core could more easily snap off than at its distal end. This would leave the collar to detach itself from the head, carrying the small plug of protein from the point where the tail penetrates the head capsid. While this argument is rather speculative it does support the contention that the hexagonal discs are collars.

Apart from purely morphological considerations, particularly useful evidence is

provided by the dimensions of the two components, both on the phage and in a free state. The results of this are summarized in Table 3 and include measurements from published micrographs taken by other authors. It can be seen that the supposed base-plates of Brenner & Horne (1959) and Anderson & Stephens (1964) are nearly the same size as the hexagonal disc in Pl. 4, fig. 2 (420 Å). The stars of Fernandez-Moran (1962), Anderson & Stephens (1964) and Pl. 3, fig. 20 (Pl. 3, fig. 19 is clearly distorted and gives a low value) are all about 600 Å, much bigger than the previous value. The most pertinent comparisons are between the components in the free state and on the intact virion. The average diameter of objects 1, 2 and 3 in Table 3 is about 370 Å, and the average for collars on intact virions is about 360 Å, but the average for base-plates is only 305 Å. Since the hexagonal discs (objects 1, 2, 3) show no signs of gross distortion or break-up, it seems that the discs correspond to collars rather than to base-plates, as postulated by these authors. While the differences are small, amounting to 2 mm. on micrographs included here, unbiased measurement shows that they are real and significant.

In summary there is no doubt from the above evidence that hexagonal discs similar to Pl. 4, fig. 23, are collars. The study and comparison of many electron micrographs provides no evidence to the contrary. The collar has long been an object of some speculation, but it can now be said that its appearance is as expected, with a sixfold radial symmetry corresponding to that of the rest of the tail. A particular point of interest is the nature of the hollow apical subunits. They can, incidentally, be seen on the intact virion in Pl. 2, fig. 10 (arrowed). It is here that the tail fibres can be released or attached by the presence or otherwise of the necessary cofactors for adsorption. Therefore this element plays an important part in the adsorption process far removed though it is from the tip of the tail. It is a remarkably thin unit being only 15 Å thick, yet it is quite stable though it frequently disappears on intact virions, presumably by flattening itself against the head.

The base-plate plays a more important part in the infective process and it appears to be a rather unstable structure, readily breaking down into the star. It is not clear how this comes about or whether it is a natural process associated with cell wall penetration.

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REFERENCES

ANDERSON, T. F. (1960). On the fine structure of the temperate bacteriophages P1, P2 and P22. *Proc. Eur. Reg. Conf. electron microscopy*, Delft, 1960, **2**, 1008.

ANDERSON, T. F. & STEPHENS, R. (1964). Decomposition of T6 bacteriophage in alkaline solutions. *Virology*, **23**, 113.

BRADLEY, D. E. (1962). A study of the negative staining process. *J. gen. Microbiol.* **29**, 503.

BRADLEY, D. E. (1963). The structure of coliphages. *J. gen. Microbiol.* **31**, 435.

BRADLEY, D. E. (1964). The structure of some bacteriophages associated with male strains of *Escherichia coli*. *J. gen. Microbiol.* **35**, 471.

BRENNER, S. & HORNE, R. W. (1959). The structure of bacteriophage. *The Times Science Review*, London, Autumn, 1959.

BRENNER, S., STREISINGER, G., HORNE, R. W., CHAMPE, S. P., BARNETT, L., BENZER, S. & REES, M. W. (1959). Structural components of bacteriophage. *J. molec. Biol.* **1**, 281.

CUMMINGS, D. J. & KOZLOFF, L. M. (1960). Biophysical properties of bacteriophage T2. *Biochem. biophys. acta*, **44**, 445.

CUMMINGS, D. J. & KOZLOFF, L. M. (1962). Various properties of the head protein of T2 bacteriophage. *J. molec. biol.* **5**, 50.

EISERLING, F. A. & BOY DE LA TOUR, E. (1964). Capsomeres and other structures observed on some bacteriophages. *Pathologia microbiologia* (in the Press).

EPSTEIN, R. H., BOLLE, A., STEINBERG, C. M., KELLENBERGER, E., BOY DE LA TOUR, E., CHEVALLEY, R., EDGAR, R. S., SUSMAN, M., DENHART, G. H. & LIELAUSIS, A. (1964). Physiological studies of conditional lethal mutants of bacteriophage T4D. *Cold Spr. Harb. Symp. Quant. Biol.* **28**, 375.

FAVRE, R., BOY DE LA TOUR, E., KELLENBERGER, E. & SÉCHAUD, J. (1964). Morphogenèse de la tête du bactériophage T4: observations sur les polytêtes. *Proc. Eur. Reg. Conf. electron microscopy*, Prague, 1964, vol. B, p. 541.

FERNANDEZ-MORAN, H. (1962). New approaches in the study of biological ultrastructure by high resolution electron microscopy. *Symp. Internat. Soc. Cell Biol.* (Ed. R. J. Harris), **1**, 411.

FINCH, J. T. (1964). The resolution of the substructure of tobacco mosaic virus in the electron microscope. *J. molec. Biol.* **8**, 872.

KLEINSCHMIDT, A. K., LANG, D., JACHERTS, D. & ZAHN, R. K. (1962). Darstellung und Längenmessungen des gesamten desoxyribonucleinsäure-Inhaltes von T2-bakteriophagen. *Biochim. biophys. acta*, **62**, 857.

MARKHAM, R., FREY, S. & HILLS, G. J. (1963). Methods for the enhancement of image detail and accentuation of structure in electron microscopy. *Virology*, **20**, 88.

WILLIAMS, R. C. & FRASER, D. (1953). Morphology of seven T bacteriophages. *J. Bact.* **66**, 458.

EXPLANATION OF PLATES

PLATE 1

Fig. 1. Phage T2 in uranyl acetate with EDTA, × 230,000.

Fig. 2. Phage T2 head in ammonium carbonate and sodium molybdate, × 200,000.

Fig. 3. Phage T2 in uranyl nitrate; 'high-and-dry' particles in profile, × 200,000.

Fig. 4. Model of proposed figure for T2 type heads in different orientations.

Figs. 5, 6. Phage T2 heads in uranyl acetate with EDTA with superimposed wire model, × 380,000.

Fig. 7. Phage T2 head in uranyl acetate with EDTA × 380,000.

PLATE 2

Figs. 8, 9. Phage T4 heads in uranyl formate, × 400,000.

Fig. 10. Phage T4 in ammonium carbonate and sodium molybdate, × 300,000.

Fig. 11. A head of phage T4 treated with anti-T4 serum in phosphotungstate, × 550,000.

Fig. 12. Polyhead (A), polysheath (B) and intact virion (C) from phage PVL in phosphotungstate, × 50,000.

Fig. 13. The components in Fig. 12, × 333,000.

PLATE 3

Fig. 14. Polyhead from phage PVL, phosphotungstate, × 333,000.

Fig. 15. Marked area of Fig. 14 printed by rotation; six exposures in one revolution, × 333,000.

Fig. 16. Polyhead from PVL showing hexagonal subunits (arrowed), phosphotungstate, × 333,000.

Fig. 17. Core from phage BP/4 in phosphotungstate, × 333,000.

Fig. 18. Phage WB/5 showing detail of base-plate, phosphotungstate, × 333,000.

Fig. 19. Partly disrupted base-plate of phage BP/4, phosphotungstate, × 333,000.

Fig. 20. Base-plate of phage BP/4 fully disrupted to star shape, phosphotungstate, × 333,000.

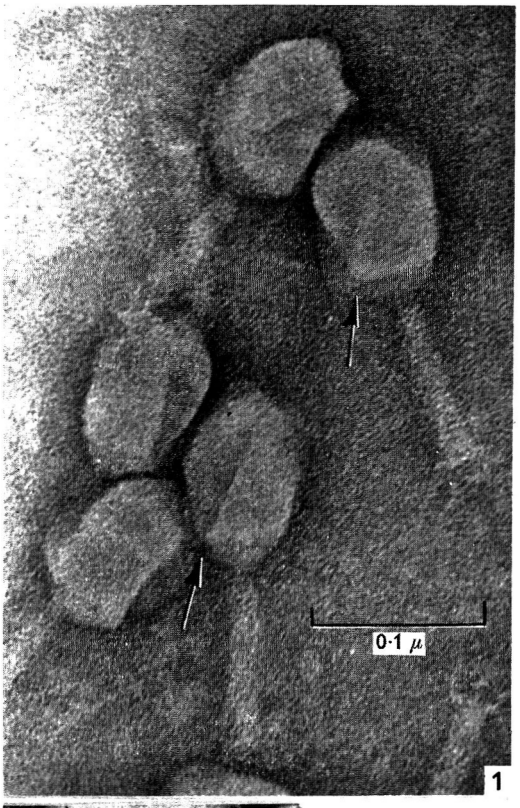
PLATE 4

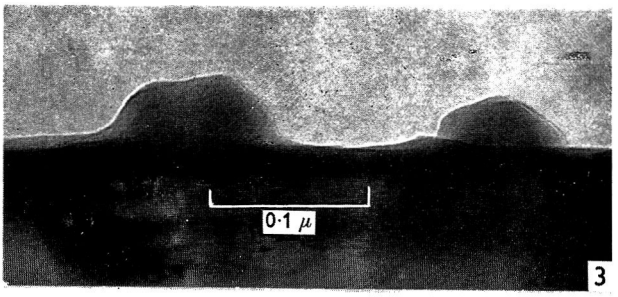
Fig. 21. Star-shaped base-plate in silicotungstate after Anderson & Stephens (1964), with permission of Virology, Academic Press Inc., × 300,000.

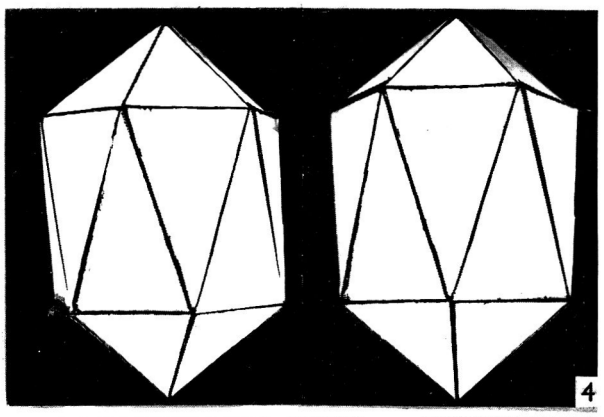
Fig. 22. Phage T4 in potassium phosphomolybdate showing collar fitting to flattened apex of basal pyramid, × 333,000.

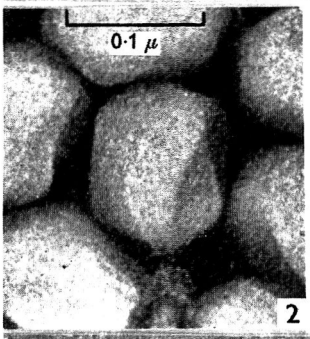
Fig. 23. Isolated collar of phage T4 in ammonium carbonate and sodium molybdate, × 300,000.

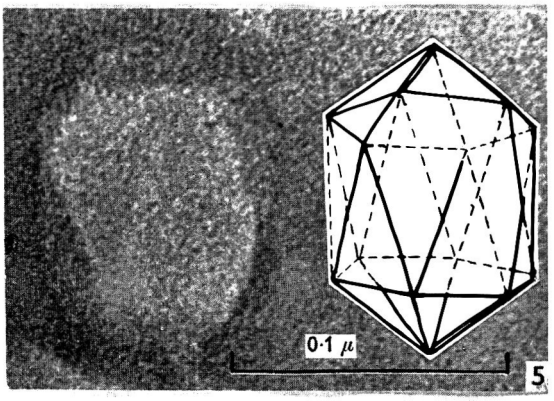
Fig. 24. Fig. 23 printed by rotation: six exposures in one revolution, × 450,000.

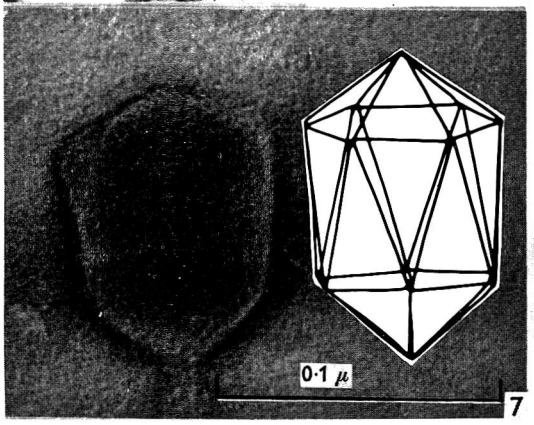


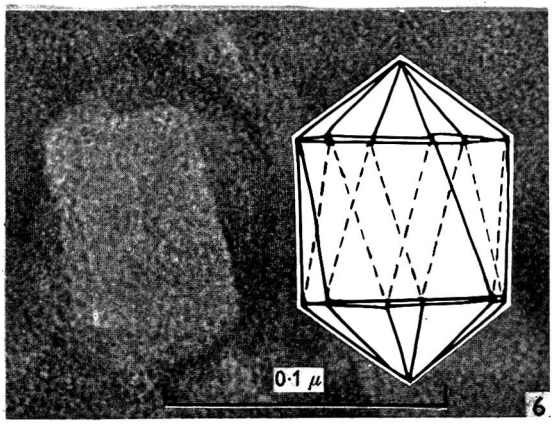












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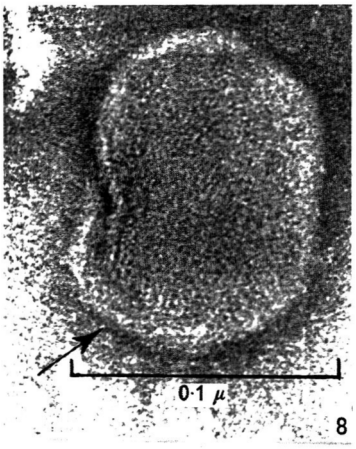
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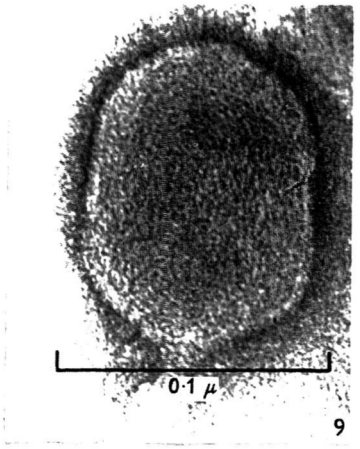
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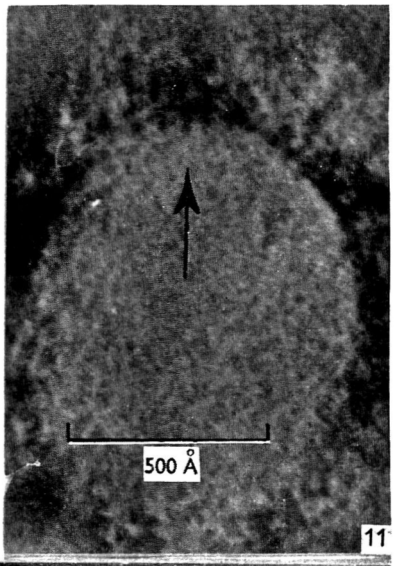
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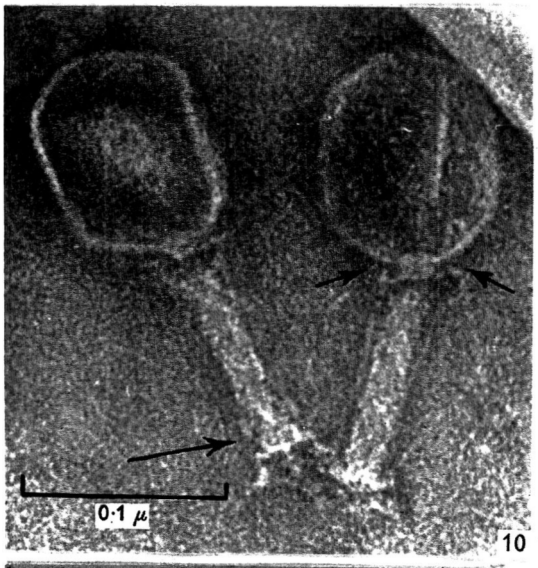
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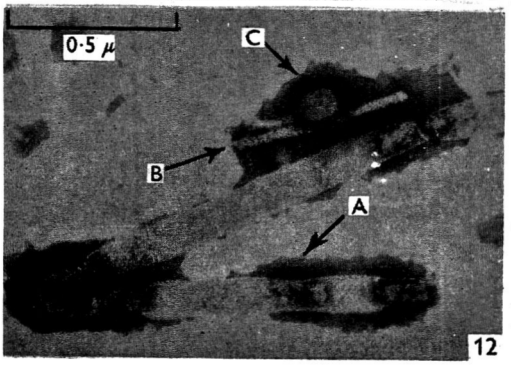
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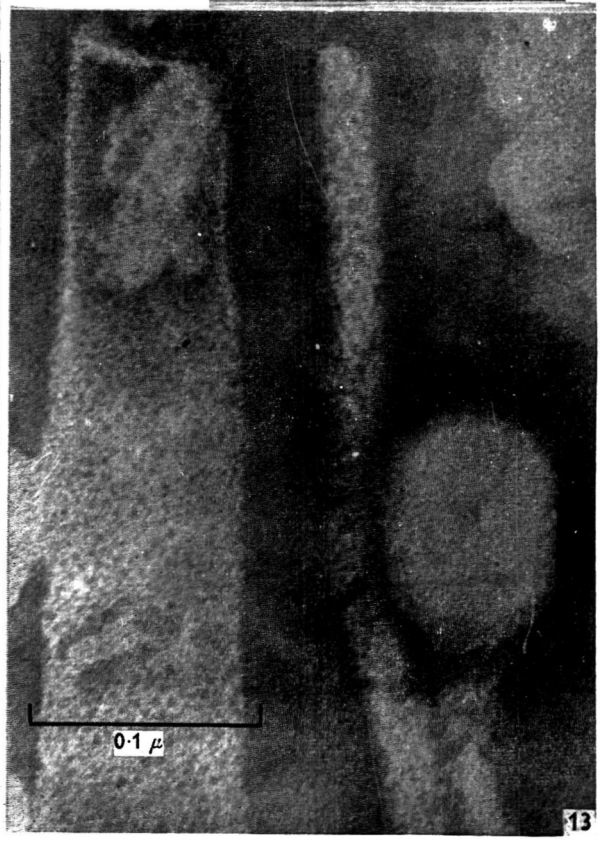
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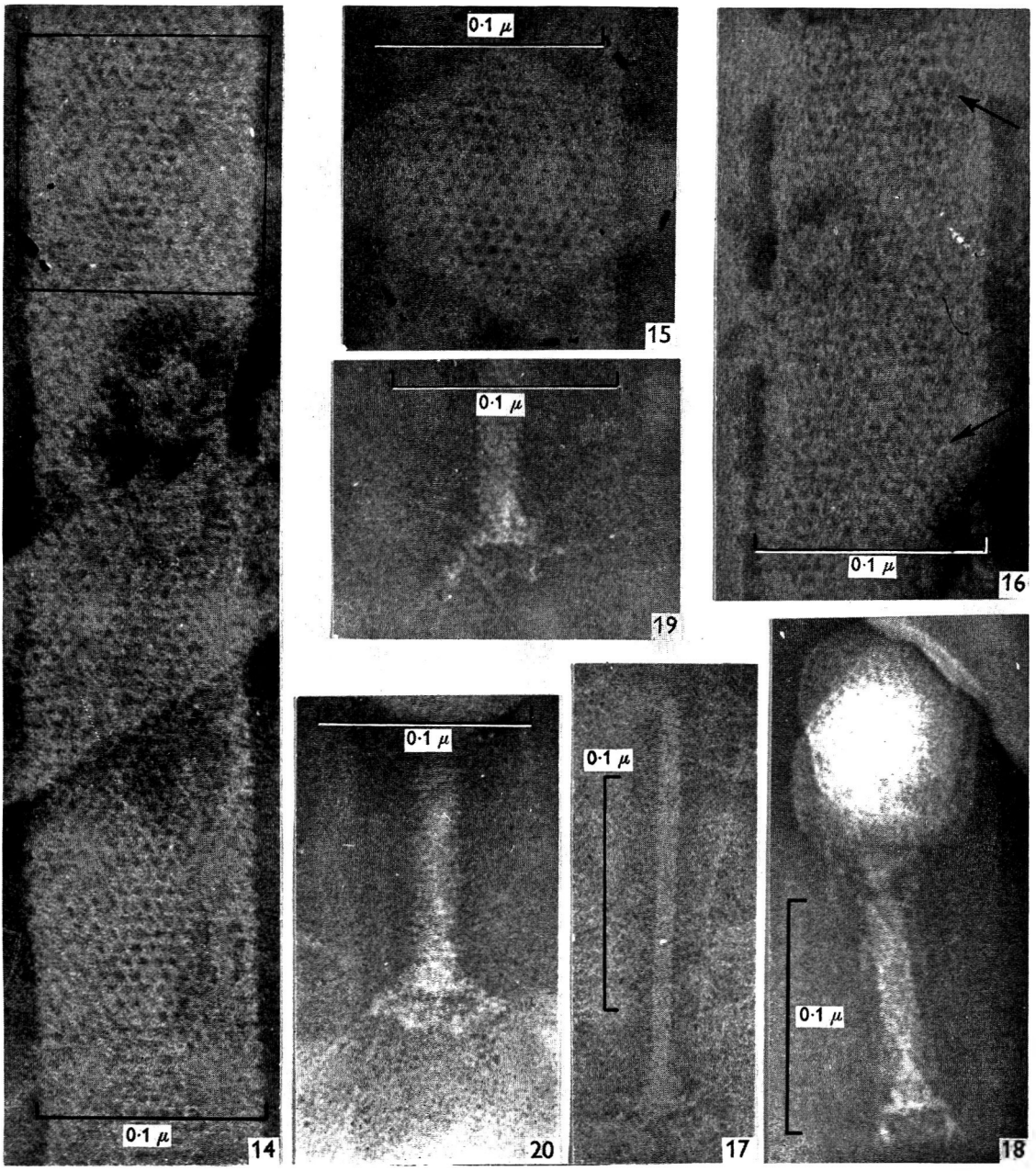
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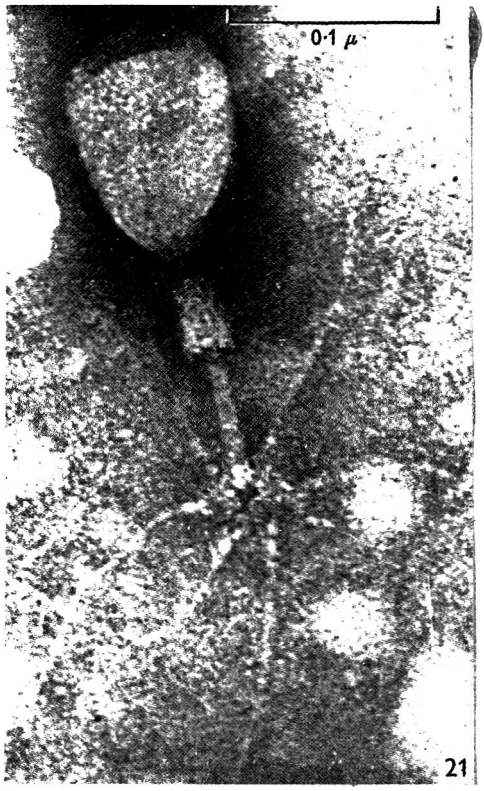
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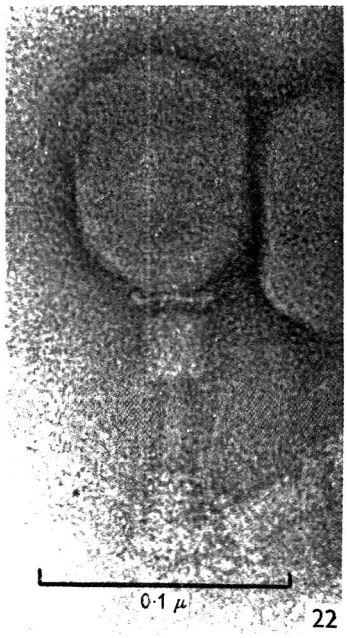
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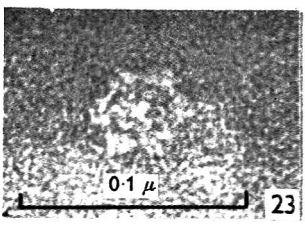
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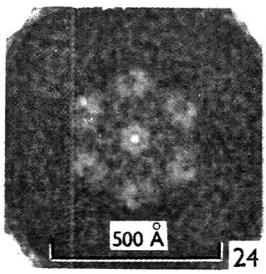
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Contamination of Rhinovirus Seed Pools Revealed in HEp2 Cell Suspension Cultures

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SUMMARY

Two seed pools of rhinovirus were contaminated accidentally at different times with the same virus. This contamination was detected only in certain 'spinner flask' experiments under circumstances which suggested the possibility that a mechanism other than contamination was responsible for the appearance of this virus. Experiments were made to test some of these possible mechanisms, but the results strongly indicated a contamination. The circumstances under which this contamination probably occurred are discussed.

INTRODUCTION

The rhinoviruses (Andrewes, 1961–62) are a large group of viruses that have many properties of enteroviruses and have been causally associated with common colds (Tyrrell *et al.* 1960; Bynoe *et al.* 1961; Hamre & Procknow, 1961; Reilly *et al.* 1962; Bloom *et al.* 1963). Rhinovirus strains have been classified into two subgroups depending on whether they grow only in cultures of human kidney cells (H strains) or also in monkey kidney cell cultures (M strains). HGP strain of rhinovirus is a prototype of M strains. In an attempt to obtain high-titred seed pools of rhinovirus, HGP was inoculated into HEp2 cell suspension cultures. During these experiments a second virus appeared and displaced the rhinovirus from these cultures. This occurred on four occasions under circumstances which suggested the possibility that a mechanism other than contamination was responsible for the appearance of this second virus. This second virus was called spinner virus because of its association with cell cultures maintained in suspension in 'spinner flasks'. Experiments were made to identify spinner virus and to determine its source. In view of the ever increasing number of recoverable viruses and of laboratories in which multiple agents are under simultaneous study, the experiments that are the subject of this report would seem increasingly pertinent.

METHODS

Viruses. The viruses used in HEp2 cell suspension experiments are listed in Table 1. Virus 1 was isolated in human embryonic kidney cell cultures in Salisbury, England, from the nasal washings of a patient with a common cold. It was then passed 4 more times in similar cultures and once in Rhesus monkey kidney cell cultures before being sent to this laboratory. Virus 2 was similarly isolated and

passed twice in human embryonic kidney cell cultures in Salisbury. Viruses 3 and 4 were serologically identified as HGP in England but had a different source or a different passage history from those of viruses 1 and 2. Virus 5 represented the eighth passage in human embryonic kidney cell cultures of virus 2. Viruses 6 and 7 were originally obtained from Salisbury and adapted to KB cells by Dr W. S. Jordan.

Table 1. *Viruses used in experiments with HEp2 cell suspensions*

Virus	Obtained from	Supplier's designation
1. HGP	Dr D. A. J. Tyrrell	cc 287
2. HGP	Dr D. Taylor-Robinson	cc 803, KELLY, (33–48), 7-2-61
3. HGP	Dr D. Taylor-Robinson	cc 671, Harrison, 1-30-61
4. HGP	Dr D. Taylor-Robinson	cc 1050, 12-14-16
5. HGP	Dr D. Taylor-Robinson	KELLY, cc 1322, 1-23-62
6. HGP	Dr W. S. Jordan	6th KB, 7-31-62
7. FEB	Dr W. S. Jordan	2nd KB, 7-31-62
8. 2060	Dr H. S. Ginsberg	TC 816, Group 2, 11-27-58
9. Measles	Dr J. F. Enders	Edmonston, 29th HK pass

Media. The growth medium used for the propagation of HEp2 cells consisted of Mixture 199 (Morgan, Morton & Parker, 1950) supplemented with 10% (v/v) calf serum.

The maintenance medium for the cells was Hanks' balanced salt solution supplemented with 2% (v/v) calf serum, 0·25% (w/v) lactalbumin hydrolysate, and 0·052% (w/v) $NaHCO_3$. All media contained 100 units of pencillin G/ml. and 100 μg. streptomycin/ml. At monthly intervals kanamycin (50 μg./ml.) and amphotericin B (2·5 μg./ml.) were added to both growth medium and maintenance medium.

Cells. The strain of HEp2 cells used was obtained from a commercial source (Microbiological Associates, Inc., Bethesda, Md., U.S.A.) and was maintained in serial culture in 32 oz. prescription bottles. Transfer of cells was made by treating with Puck's saline A (Puck, Cieciura & Fisher, 1957) and then 0·04% (w/v) trypsin (1/250, Difco) in saline A to detach them from the glass. Test-tube cultures were prepared by the inoculation of screw-capped tubes with approximately 60,000 cells suspended in 0·5 ml. growth medium.

In preparing spinner flask cultures, trypsin-treated cells were resuspended in maintenance medium rather than in growth medium and the cell concentration was adjusted to 1×10^5 to 6×10^5 cells/ml. Although suspended in maintenance medium, the number of cells generally doubled during the first 24 hr of incubation at 33° but did not increase after this.

Preparation of rabbit antisera. Young male albino rabbits were inoculated (4 to 6 times) intravenously at 7- to 10-day intervals with 3–5 ml. of infectious virus suspension. There were no signs of illness following these inoculations. Rabbits were bled initially and the sera were tested for antibodies before each inoculation after the third. Generally neutralizing antibody titres of 1000 against 100 TCID 50 (signifies 50% tissue culture infectious doses) of virus were achieved. After three intravenous inoculations one rabbit was given a subcutaneous injection consisting of 1·5 ml. of spinner virus with a titre of 10^8 TCID 50/0·1 ml. and 3·5 ml. of complete Freund's adjuvant. Serum from this rabbit also achieved maximal titres of

neutralizing antibody of 1000. Typing antisera for Coxsackie types A 1–8, A 10–24 and B 1–6, and for echovirus types 29–32 were kindly supplied by Dr H. A. Wenner.

Neutralization tests. Equal volumes of virus suspensions containing 100 TCID 50/0·1 ml. and two-fold serum dilutions were mixed and incubated for 3 hr at 37°. For each serum dilution 0·2 ml. of this mixture was inoculated into each of four HEp2 cell tube cultures freshly changed with 1·0 ml. maintenance medium. These tubes were incubated for 5 days in a roller wheel at 33° without changing medium. They were then examined for cytopathic effects and the neutralizing antibody titres were determined. The neutralization tests with spinner virus and antisera specific for poliovirus types 1, 2 and 3, Coxsackie viruses types A 9 and B 1–5, and echoviruses types 1–27 were done by Dr Martha Lepow in Rhesus monkey kidney cell cultures.

Complement-fixation test. Complement-fixation tests were made by a modification of the drop method of Fulton & Dumbell (1949), with 2·5 units of complement.

Virus titrations. Virus from infected tube cultures consisted of pooled tissue-culture medium. Virus from spinner flask cultures was obtained by freezing and thawing the cell suspension 3 times and centrifuging to remove cell debris. Virus titrations were done in HEp2 cell tube cultures incubated at 33° in a roller wheel. Serial 10-fold dilutions of virus were prepared in maintenance medium and 0·1 ml. of each dilution was inoculated into 3 HEp2 cell tube cultures. The cultures were examined for cytopathic effects and the medium changed every 48 hr for a total of 6 days, at which time titrations of spinner virus were discarded. Titrations of rhinovirus were kept for 8 days. On the final day HEp2 cell culture tubes were scored for the presence or absence of cytopathic effects and the 50% end-point was calculated by the method of Reed & Muench (1938).

Incubation conditions. Unless otherwise specified, infected tube cultures were incubated at 33° in a roller wheel and spinner flask cultures at 33°.

Stains. Acridine orange staining was done on coverslip cultures according to the method of Bertalanffy (1958). Haematoxylin and eosin stains were done on cells fixed in Bouin's solution.

Other tests. Chloroform sensitivity tests were done by the method of Feldman & Wang (1961). Tests of stability at pH 5 and 60° were done as outlined by Dimmock & Tyrrell (1962). The ability of 5-fluoro-2′-deoxyuridine (FUDR) to inhibit viral replication was determined according to the method of Salzman (1960). Herpes simplex virus and Coxsackie B, Type 3 virus were used as controls in these experiments.

RESULTS

The experiments with HEp2 cell suspensions were made to determine whether or not the HGP virus would grow to higher titres in suspended cells than in tube cultures. The initial results showed that the titre of virus was higher in spinner flasks of suspended cells and that the cytopathic effect was indistinguishable from that of HGP virus. It was not then apparent that another virus might be responsible for these results. After antisera were prepared against spinner virus, it was found that there was no serological relationship between spinner virus and HGP virus, and that all four of the spinner virus strains were serologically identical. The

possibility was then explored that spinner virus was a contaminant. In support of this hypothesis was the fact that in each experiment in which spinner virus appeared, the HGP virus used could be traced to either a specific seed pool of virus 1 or a similar pool of virus 2 (Table 1). The contamination hypothesis was initially rejected for several reasons. First, the seed pool of virus 1 had been stored before virus 2 was introduced into the laboratory. Secondly, the only virus similar to spinner virus in the laboratory when these pools were in preparation was a stool isolate present only during the formation of the pool of virus 2. An attempt had been made to isolate virus from this stool during the preparation of the pool of virus 1, but had been unsuccessful. The third reason for initially rejecting the contamination hypothesis was that although the pools of virus 1 and 2 had been inoculated into tube cultures of HEp2 cells on many occasions, spinner virus was not detected. Although spinner virus grows very well in tube cultures, it was exclusively associated with HEp2 cell suspensions.

The possibility was next investigated that the cells might themselves be the source of this virus. Uninfected HEp2 cell suspension cultures were prepared and studied in the same way as infected cultures, but no spinner virus was detected. Material from frozen and thawed uninfected cells was passed serially into suspension cell cultures but no virus appeared. Then the possibility was tested that a virus infection was necessary to 'induce' the appearance of spinner virus which was latent in the HEp2 cells. This was done by inoculating viruses 3 to 9 (Table 1) into HEp2 cell suspensions. Material from these frozen and thawed infected cultures was then passed serially into suspension cell cultures; but spinner virus was not detected.

The HGP seed pools associated with spinner virus were then considered as a possible source. This was tested by an experiment to see whether treatment with spinner antiserum could 'cure' of spinner virus contamination a sample of a seed pool of HGP virus known to be associated with spinner virus in HEp2 cell suspension cultures. A sample of an appropriate HGP seed pool was selected and passaged serially 3 times at its terminal dilution in HEp2 cell tube cultures in the presence of sufficient spinner antiserum to neutralize 1000 TCID 50 of virus. Treated virus was then tested for spinner virus by serial passage into HEp2 cell suspension cultures, but no spinner virus was found. A sample of treated virus was passaged serially 10 more times without spinner antiserum in HEp2 cell tube cultures. When this virus was tested for spinner virus in suspension cell cultures, none was found. This result was inconclusive, however, because untreated samples of virus from pools associated with spinner virus were frequently also negative when similarly tested.

Another aspect of the contamination hypothesis of the origin of spinner virus was studied as follows. Two pools of HGP virus were deliberately contaminated with spinner virus, one with the virus at its terminal dilution and one at 0·5 $\log_{10}$ unit beyond the terminal dilution. These contaminated pools were then serially passaged in HEp2 cell tube cultures. After three passages, spinner virus was detected in all tubes of this experiment. In the light of this result, the repeated failure to detect spinner virus in tube cultures inoculated with pools known to be associated with spinner virus was interpreted as evidence against the contamination hypothesis.

Experiments to characterize and identify spinner virus were then made in the hope that they would give data from which other hypotheses of its origin might be

derived. Some of these data (summarized in Table 2) indicated that spinner virus was an enterovirus. Dr Martha Lepow attempted unsuccessfully to identify spinner virus with antisera specific for the enterovirus serotypes likely to be encountered in tissue cultures. Subsequent tests with antisera specific for the Coxsackie A types not likely to be isolated in tissue culture also failed to identify spinner virus.

Table 2. *Properties of spinner virus and HGP virus*

Property	HGP	Spinner virus
1. Contains RNA	+	+
2. Ether and chloroform resistant	+	+
3. Enterovirus-like cytopathic effect in human and monkey cells	+	+
4. Cytopathic effects and titre in tube cultures enhanced by rolling	+	+
5. Stable indefinitely at −70°	+	+
6. Stable for 10 days at 20–23°	−	+
7. Stable at pH 5·0	−	+
8. Stable at 60° for 1 hr in M-$MgCl_2$	−	+

+ indicates property present; − indicates property absent.

The only other hypothesis about the origin of spinner virus which was considered was that it represented a mutant or variant of HGP virus which was selected by suspension cell culture conditions. Attempts to show a serological relationship between spinner virus and HGP virus in complement-fixation tests were unsuccessful. Because of this and the many other important biological differences between spinner virus and HGP virus summarized in Table 2 the mutation hypothesis seemed untenable and the contamination hypothesis more probable.

The stool isolate mentioned previously which had been present during the formation of the seed pool of virus 2 (Table 1) was then reconsidered as a source of this contamination. Virus from this stool was tested with spinner antiserum and was found to be serologically identical with spinner virus. Dr Martha Lepow then again attempted to identify spinner virus with enterovirus antisera and was able to show that spinner virus and the stool isolate were strains of Coxsackie B virus, type 5. These findings strongly indicated that the stool isolate was the source of spinner virus and that the HGP virus seed pools associated with spinner virus had been contaminated with it.

The stool isolate had been present in the laboratory during the preparation of the contaminated seed pool of virus 2 (Table 1). An isolation attempt in Rhesus monkey kidney cell cultures from this stool had been present during the preparation of the contaminated seed pool of virus 1 (Table 1) but at that time no virus was isolated. Rhesus monkey kidney cell cultures used in this isolation attempt were markedly affected by a simian virus infection and it is possible that stool virus was present and multiplying but a cytopathic effect not detected. It has not been possible retrospectively to identify any other opportunity for the demonstrated stool virus contamination of the virus 1 seed pool to have occurred. A subsequent attempt to isolate virus from this stool in both HEp2 cells and Rhesus monkey kidney cell was successful only in the HEp2 cells, which indicates that they were more sensitive to this virus than Rhesus monkey kidney cells under the conditions used. It has not been possible to explain the initial failure to identify spinner virus as Coxsackie B, type 5. Review of the protocol of this experiment suggested that the

technique used had influenced the results: the spinner virus used was titrated in stationary tubes and the test itself was incubated in a roller wheel. Since the titre of spinner virus was usually 10-fold higher in rotated tubes than in stationary tubes, it is possible that too much virus had been inoculated for the antiserum dilution used.

All the techniques used in the laboratory were reviewed in an attempt to determine how this contamination had occurred. It seemed most likely that it occurred during the process of changing medium in infected tube cultures. During this process the screw cap of the culture tube is held in the fifth finger of the pipetting hand while the tube itself is removed from the cap and re-inserted into it with the other hand. If the lip of a tube containing virus should touch the hand near to the screw cap being held, some virus might be deposited there and survive to be transferred to another tube which also touched the contaminated place on the hand. An experiment was made to see whether such contamination might occur even in the face of flaming the culture tubes, as was routinely done. A pipette was dipped into a suspension of spinner virus with a concentration of 10^5 TCID 50/0·1 ml. and the tip of the pipette then touched to the hand near where screw caps are ordinarily held. Twelve uninfected HEp2 cell tube cultures were in turn uncapped and the lip of the tube touched to the deliberately contaminated area of the hand. The first six of these tubes were flamed in the usual way and the last six were not flamed. All twelve of the tubes showed spinner virus cytopathic effect within 48 hr.

DISCUSSION

Contaminations in tissue culture and virology laboratories are probably not rare events. The contamination of tissue cells in continuous culture with Mycoplasma and cells of other lines has been the subject of several reports (Rothfels *et al.* 1959; Clausen & Syverton, 1962; Defendi *et al.* 1960; Robinson, Wichelhausen & Roizman, 1956; Holmgren & Campbell, 1960). Laboratory-acquired infections with viruses, including the Coxsackie viruses (Shaw, Melnick & Curnen, 1950; Shelokov & Habel, 1957; Bell & Meis, 1959) have been described, and the two individuals who worked with spinner virus in this laboratory each developed significant rises in neutralizing antibody titre against this virus. These infections were clinically inapparent, as were some of the infections described by Lerner, Klein & Finland (1960).

A review of the literature for the last 10 years revealed only one report (Siegel, 1956) of a laboratory contamination with a virus that had features similar to the contamination reported here. An important similarity is that the contaminating virus shared several biological properties with the virus that was contaminated. It was because of this fact that the contamination went unrecognized for some time.

The contaminant Coxsackie B, type 5 virus described here likewise had several biological properties very similar to those of the rhinoviruses. Another factor which contributed to its non-recognition was that the contaminant was present in only trace amounts, so that its occurrence was relatively infrequent and under such circumstances as to suggest another mechanism for its origin. The exclusive association of spinner virus with 'spinner flask' experiments seems largely to have been due to the relatively large volumes of undiluted contaminated rhinovirus pools used in

such experiments. It is estimated from the frequency with which spinner virus was detected and the total volume of the contaminated pools, that only 20% of 5-ml. samples from these pools would have contained an infective dose of the contaminant. Similarly, only 0·4% of 0·1 ml. samples (the volume routinely used in tube culture experiments) would have included an infective dose of spinner virus. It seems likely that because its cytopathic effect is grossly indistinguishable from that of HGP virus the appearance of spinner virus in an occasional tube culture would not have been noted. It is equally likely that, were it not for the 'spinner flask' experiments, the contamination would not have been detected at all.

The contamination reported here serves to re-emphasize the constant attention necessary to prevent such occurrences. It is not possible to be certain exactly how this particular contamination occurred; it may have happened when medium was being changed. This possibility is supported by the results of the experiment to contaminate culture tubes from the hand, which suggest that flaming the lips of culture tubes as done routinely in the laboratory would have been insufficient to prevent contamination. Methods designed to destroy a contaminant such as the one described here seem likely to fail often and are no substitute for keeping potential contaminants from the work area altogether.

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REFERENCES

Andrewes, C. H. (1961–62). The taxonomic position of common cold viruses and some others. *Yale J. Biol. & Med.* **34**, 200.

Bell, J. F. & Meis, A. (1959). Pericarditis in infection due to Coxsackie virus group B type 3. *New England J. Med.* **261**, 126.

Bertalanffy, L. von (1958). A rapid screening method for the detection of cancer cells. *Cancer*, **11**, 873.

Bloom, H. H., Forsyth, B. R., Johnson, K. M. & Chanock, R. M. (1963). Relationship of rhinovirus infection to mild upper respiratory disease. I. Results of a survey in young adults and children. *J. Amer. med. Ass.* **186**, 38.

Bynoe, M. L., Hobson, D., Horner, J., Kipps, A., Schild, G. C. & Tyrrell, D. A. J. (1961). Inoculation of human volunteers with a strain of virus isolated from a common cold. *Lancet*, i, 1194.

Clausen, J. J. & Syverton, J. T. (1962). Comparative chromosomal study of 31 cultured mammalian cell lines. *J. nat. Cancer Inst.* **28**, 117.

Defendi, V., Billingham, R. E., Silvers, W. K. & Moorhead, P. (1960). Immunological and karyological criteria for identification of cell lines. *J. nat. Cancer Inst.* **25**, 359.

Dimmock, N. J. & Tyrrell, D. A. J. (1962). Physiochemical properties of some viruses isolated from common colds (rhinoviruses). *Lancet*, ii, 536.

Feldman, H. A. & Wang, S. S. (1961). Sensitivity of various viruses to chloroform. *Proc. Soc. exp. Biol., N.Y.* **106**, 736.

Fulton, F. & Dumbell, K. R. (1949). The serological comparison of strains of influenza virus. *J. gen. Microbiol.* **3**, 97.

Hamre, D. & Procknow, J. J. (1961). Viruses isolated from natural common colds in the U.S.A. *Brit. med. J.* **11**, 1382.

HOLMGREN, N. B. & CAMPBELL, W. E. JR. (1960). Tissue cell culture contamination in relation to bacterial pleuropneumonia-like organisms-L form conversion. *J. Bact.* **79**, 869.

LERNER, A. M., KLEIN, J. O. & FINLAND, M. (1960). A laboratory outbreak of infections with Coxsackie virus group A, type 9. *New England J. Med.* **263**, 1302.

MORGAN, J. F., MORTON, H. J. & PARKER, R. C. (1950). Nutrition of animal cells in tissue cultures. I. Initial studies on a synthetic medium. *Proc. Soc. exp. Biol. Med.* **73**, 1.

PUCK, T. T., CIECIURA, S. J. & FISHER, H. W. (1957). Clonal growth *in vitro* of human cells with fibroblastic morphology. *J. exp. Med.* **106**, 145.

REED, L. J. & MUENCH, H. (1938). A simple method of estimating fifty per cent end points. *Am. J. Hyg.* **27**, 493.

REILLY, C. M., HOCH, S. M., STOKES, J. JR., MCCLELLAND, L., HAMPARIAN, V. V., KETLER, A. & HILLEMAN, M. R. (1962). Clinical and laboratory findings in cases of respiratory illness caused by coryzaviruses. *Ann. int. Med.* **57**, 515.

ROBINSON, L. B., WICHELHAUSEN, R. H. & ROIZMAN, B. (1956). Contamination of human cell cultures by pleuropneumonia-like organisms. *Science*, **124**, 1147.

ROTHFELS, K. H., AXELROD, A. A., SIMINOVITCH, L., MCCULLOCK, E. A. & PARKER, R. C. (1959). The origin of altered cell lines from mouse, monkey and man as indicated by chromosome and transplantation studies. *Proc. 3rd Canad. Cancer Conf.* pp. 189–214. New York: Academic Press Inc.

SALZMAN, N. P. (1960). The rate of formation of vaccinia deoxyribonucleic acid and vaccinia virus. *Virology*, **10**, 150.

SHAW, E. W., MELNICK, J. L. & CURNEN, E. C. (1950). Infection of laboratory workers with Coxsackie viruses. *Ann. int. Med.* **33**, 32.

SHELOKOV, A. & HABEL, K. (1957). Viremia in Coxsackie B meningitis. *Proc. Soc. exp. Biol., N.Y.* **94**, 782.

SIEGEL, B. V. (1956). Contaminant filterable agent derived from a human wart. *J. invest. Derm.* **27**, 379.

TYRRELL, D. A. J., BYNOE, M. L., HITCHCOCK, G., PEREIRA, H. G., ANDREWES, C. H. & PARSONS, R. (1960). Some virus isolations from common colds. *Lancet*, i, 235.

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Serological Specificities of Ureases of Proteus Species

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SUMMARY

Ureases of *Proteus vulgaris*, *P. mirabilis and P. rettgeri* were found to be serologically indistinguishable, while that of *P. morganii* was distinct from the other three. This finding, together with recent observations on the phage susceptibilities and base ratios of the nucleic acids of these organisms, would seem to indicate that *P. mirabilis* and *P. rettgeri* are merely variants of *P. vulgaris*, while *P. morganii* is a distinct species.

INTRODUCTION

Biochemical reactions have been used in the speciation of bacteria, particularly in the field of enteric bacteriology, where the concept of a species is almost exclusively established on the basis of biochemical pattern. Evidence has accumulated which indicates that the syntheses of bacterial enzymes which carry out biochemical reactions are not always stable and the difference between positive and negative reactions in biochemical tests may not have as much significance as was once believed. For example, with tryptophan synthetases of *Neurospora crassa* and *Escherichia coli*, Yanofsky (1960) found that there were many mutants which did not synthesize this enzyme complex although the parent strains did so. Some mutants of *N. crassa* which did not produce this enzyme were able to form a protein at least immunologically related to the enzyme, whereas a second group lacked any trace of this substance (Suskind, Yanofsky & Bonner, 1955). A similar observation has been made about lecithinase of *Pseudomonas fluorescens* (Bates & Liu, 1963) and a complementation phenomenon by lecithinase-negative strains to form an active complex of the enzyme was demonstrated. This suggested that the determination of the serological specificity of an enzyme or its antigenic subunit might be a more reliable method to establish the relationship of bacterial strains than the demonstration of positive or negative reactions in biochemical tests. In this laboratory, species-specific extracellular antigens (including enzymes) have been demonstrated in the genera *Pseudomonas*, *Aeromonas*, *Serratia* and in other Gram-negative bacteria, and the specificities of these antigens were found useful as criteria in speciation of these organisms (Liu, 1961*a*, *b*; Esselmann & Liu, 1961).

Urease production is a prominent characteristic of the genus *Proteus*. It has been used as a criterion in both diagnostic and systematic work to identify and differentiate Proteus from other non-lactose-fermenting pathogens. Since the classification of species within this genus is based solely upon their biochemical reactions, the relationships of the Proteus species are obscure. The present communication describes observations on the serological specificities of the ureases of Proteus species.

METHODS

The organisms used, their sources and biochemical patterns are listed in Table 1.

Extraction and titration of urease. An organism was grown on brain heart infusion agar (Difco) containing 10% (v/v) human blood, 2·0% (w/v) urea and 2·5% (v/v) glycerol at 22–25° for 24 hr. The bacteria were washed off with saline, centrifuged at 14,000*g* for 1 hr and the supernatant fluid discarded. For *Proteus morganii* this process was repeated to wash the organisms, but other species were not so washed because this considerably decreased the titres of urease that was extracted.

Table 1. *Sources and biochemical reaction of Proteus species used*

Species	Strain	Sources	Glucose	Lactose	Maltose	Sucrose	Mannitol	Urease	Indole	Gelatin liquef.	H_2S
P. vulgaris	13315	ATCC*	AG	–	+	+	–	+	+	+	+
	4210–62	CDC†	AG	–	+	+	–	+	+	–	+
	1	CH‡	A	–	+	+	–	+	+	+	+
	5	CH	AG	–	+	+	–	+	+	+	+
P. mirabilis	402–62	CDC	AG	–	–	D	–	+	–	+	+
	668–64	CDC	AG	–	–	D	–	+	–	+	+
	JH	JH§	AG	–	–	D	–	+	–	+	+
P. morganii	6879–61	CDC	AG	–	–	–	–	+	+	–	–
	252–64	CDC	AG	–	–	–	–	+	+	–	–
	10	CH	AG	–	–	–	–	+	+	–	–
P. rettgeri	7012–61	CDC	A	–	–	–	+	+	+	–	–
	816–63	CDC	AG	–	–	–	+	+	+	–	–
	13	CH	A	–	–	–	+	+	+	–	–

AG = acid with gas formation; + = positive reaction; – = negative reaction; D = delayed reaction.
* ATCC = American Type Culture Collection; † CDC = Communicable Disease Center, Atlanta, Ga., U.S.A.; ‡ CH = Children's Hospital, Louisville, Ky., U.S.A.; § JH = Jewish Hospital, Louisville, Ky., U.S.A.

About 10 g (wet weight) of organism were suspended in 50 ml. saline; 10 ml. of a solution consisting of one volume of *n*-amyl alcohol and four volumes of chloroform was added to this suspension. Effective extraction of the urease was achieved at room temperature by forcing the bacterial suspension through a narrow aperture by repeated pipetting, thus achieving thorough mixing. The mixture was then allowed to stand at 4° for 24 hr. An exception was in the case of *Proteus morganii*, which required 2–4 days for sufficient extraction of the enzyme. The supernatant fluid (water layer) was collected by centrifugation at 14,000*g* at 4° for 1 hr.

The aqueous fluid thus obtained was sterile except in the case of *Proteus morganii* extracts, which had to be filtered through a sintered-glass filter to sterilize. The enzymic titre was decreased to 50% by this additional filtration. Since the ureases are very sensitive to heavy metal ions (Sumner, 1928) no preservative containing such ions, e.g. thiomersalate, was used.

Serial twofold dilutions of extract were made in saline containing 1/8 dilution of normal rabbit serum which served as a stabilizer of the urease. An equal volume of urea reagent (containing %, w/v: 2·0, urea; 0·91, KH_2PO_4; 0·95, Na_2HPO_4; 0·005, phenol red) was added and the mixture incubated at 37° for 24 hr. The reciprocal of

the highest dilution which showed a complete change of the colour of the indicator as the result of ammonia production was taken as the titre of the preparation. Two controls were used: one contained only diluent and urea reagent; the other consisted of the urease preparation and urea reagent but without urea. One unit of urease is defined here as the amount of the enzyme able to decompose urea 10 mg./ml., as shown in the aforementioned colour reaction. One unit of the jack bean urease (Nutritional Biochemical Co., Cleveland, Ohio, U.S.A.) as defined by Sumner (1926) was equivalent to 256 units in our system.

Production of antisera. For immunization, crude urease preparations of *Proteus vulgaris* (13315), *P. mirabilis* (402–62, and JH) and *P. rettgeri* (7012–61) were precipitated in the cold with 3 volumes of 95% (v/v) ethanol in water at pH 7·0, and the precipitates collected immediately by centrifugation at 1300*g* for 10 min. at −10°. Saline containing 1/8 dilution of normal rabbit serum was pre-cooled to 0° and used to dissolve the precipitates to the original volume. This preparation was dialysed against phosphate buffer (pH 7·2) at 4° for 2 days, with frequent changes of buffer.

The urease of *Proteus morganii* appeared to be more stable than that of the other species examined. The crude enzyme preparation was deproteinized by the addition of an equal volume of a chloroform + *n*-amyl alcohol mixture (Meyer & Chaffee, 1941). The mixture was centrifuged at 1300*g* at 4° for 20 min. and the aqueous phase, containing all of the urease activity, precipitated with 3 volumes of ethanol. The precipitate was dissolved in saline, $\frac{1}{4}$ of the original volume, and dialysed against phosphate buffer. The addition of serum as stabilizer was not necessary.

Samples of the extracts thus obtained (1·0 ml.) were mixed with equal volumes of Freund's incomplete adjuvant (Arlacel and Bayol in the ratio of 1·5/8·5) and two doses were given intradermally one week apart to rabbits. These intradermal injections were usually followed by intravenous injection of the antigens without the adjuvant in 3rd and 4th weeks. However, it was found that the intraperitoneal injection of the urease + antiserum complex as described by Kirk & Sumner (1931) gave better results. After 2 weeks the rabbits were bled and 1 ml. of the serum was mixed with an equal volume of urease preparation and injected into the same animal intraperitoneally. This injection was made twice a week during the 3rd and 4th weeks. The rabbits were bled one week after the last injection and the sera were sterilized by filtration through Millipore filters (Fig. 1). As in the case of urease preparations no preservative were used for these antisera. With this procedure antisera to the ureases of *Proteus vulgaris* (13315), *P. morganii* (6879–61) and jack

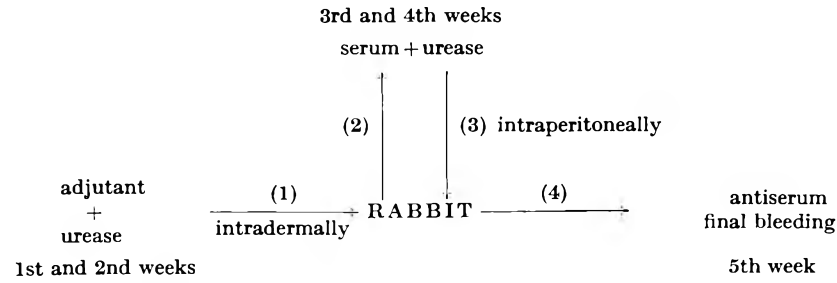

Fig. 1. The scheme for immunization of rabbits against urease.

bean were produced. However, rabbits did not respond to the ureases of *P. mirabilis* (402–62, JH) and *P. rettgeri* (7012–61) in spite of repeated attempts. Consequently, chickens were used. About 30 units of these ureases without rabbit serum were injected subcutaneously at weekly intervals for 5 weeks, the animals bled 8 days after the last injections.

Neutralization test. Kirk & Sumner (1934) reported that antibody against jack bean urease precipitated the enzyme but did not inhibit its activity. A similar phenomenon was noted with ureases of Proteus species and their antisera. The activities of antisera were, therefore, estimated by the removal of urease by precipitation. A sample (0·5 ml.) of urease preparation was added to an equal volume of antiserum, incubated at 37° for 1 hr, and the precipitate removed by centrifugation. The residual urease activity in the supernatant fluid was titrated in the usual way. A sample of homologous normal serum obtained from each animal before immunization was used as control. In testing the precipitating activity of chicken antisera, the antigens were prepared in 8·0% NaCl solution, which was necessary for maximal precipitation in this system (Goodman, Wolfe & Norton, 1951).

RESULTS

All attempts to prepare antisera to the urease of *Proteus rettgeri* were unsuccessful.

In Table 2 are shown the results of cross-precipitation of five ureases and four antisera. Close immunological relationships among ureases of *Proteus vulgaris*, *P. mirabilis*, and *P. rettgeri* were evident. The only exception noted was that the urease of *P. vulgaris* (13315) was not precipitated by the antisera to the urease of *P. mirabilis* (JH), although the urease of strain JH was precipitated by the antisera of strain 13315. We also examined the serological specificity of the proteases of *P. vulgaris* and *P. mirabilis* strains and found them to be immunologically indistinguishable. However, protease could not be used to compare these organisms with *P. morganii* and *P. rettgeri* because the latter two were non-proteolytic.

Table 2. *Cross-precipitation of ureases and antisera of Proteus strains*

		Anti-urease serum			
Source of urease		*P. vulgaris* 13315	*P. mirabilis* JH	*P. morganii* 6879–61	Jack bean
P. vulgaris	13315	64/256*	0/128	0/256	0/256
	4210–62	4/16	2/16	0/16	0/16
	CH 1	32/256	4/128	0/256	0/256
	CH 5	4/256	2/64	0/256	0/256
P. mirabilis	668–64	16/32	8/64	0/32	4/32
	402–62	32/512	4/64	0/512	0/512
	JH	32/512	8/256	0/512	0/512
P. morganii	252–64	0/8	0/8	8/8	0/8
	6879–61	0/256	0/64	256/256	0/256
	CH 10	0/64	0/64	64/64	0/64
P. rettgeri	816–63	8/64	2/16	0/64	0/64
	7012–61	16/128	2/32	0/128	0/128
	CH 13	16/128	ND	0/128	0/128
Jack bean		0/512	0/512	0/512	32/512

* 64/256 = denominator indicates the units of urease used; numerator indicates the amount of urease removed by 0·5 ml. antisera.

The ureases of all three strains of *Proteus morganii* were immunologically indistinguishable and were distinct from those of other species. No cross-reaction was noted between jack bean urease and Proteus urease, with only one exception. The urease of *P. mirabilis* (668–64) was invariably precipitated by antiserum to jack bean urease, although the extent of precipitation was much less than that with the homologous urease.

DISCUSSION

A close relationship of *Proteus vulgaris* and *P. mirabilis* has been recognized since the work of Wenner & Rettger (1919). The present classification of four species within the genus *Proteus* was the result of a study by Rustigian & Stuart (1945). However, there was disagreement even between these two authors: Rustigian was inclined to give specific rank to *P. mirabilis* while Stuart was inclined to group the two 'types' into one species, *P. vulgaris*. Production of indole has been considered to be of some taxonomic significance and on this basis Rustigian & Stuart (1945) separated *P. mirabilis* from *P. vulgaris* as distinct species. However, recent studies on the tryptophan synthetase of *Escherichia coli* (Yanofsky, 1960) indicated that the indole production is subject to the same degree of variations as any enzyme system within the descendants of a single strain. Demonstration of the serological relationship of their ureases would support the concept that these two organisms are closely related (only variants of one species).

Proteus rettgeri is generally considered to be quite distinct from other species of the genus *Proteus* because of its ability to ferment mannitol and its failure to liquefy gelatin (Rustigian & Stuart, 1945); recent evidence, however, suggested that this organism might be closely related to *P. vulgaris* and *P. mirabilis*. Vieu (1958) reported the isolation of bacteriophages which lysed *P. vulgaris* and *P. mirabilis* as well as *P. rettgeri* but not *P. morganii*. Falkow, Ryman & Washington (1962) reported that the base composition of the deoxyribonucleic acids (DNA) of *P. rettgeri*, as expressed in terms of guanine + cytosine (G + C) content, was exactly the same as those of *P. vulgaris* and *P. mirabilis*, while that of *P. morganii* was distinct. The authors did not consider the similarity of G + C content alone as sufficient evidence to indicate that two organisms belong to the same species, because many widely different organisms can possess DNAs of similar base content. However, demonstration of the serological relationship of the urease of *P. rettgeri* with those of *P. vulgaris* and *P. mirabilis* would indicate that the structures of the genes that direct the synthesis of this enzyme in these three organisms are very similar because, if one accepts the one gene–one enzyme hypothesis, the amino acid sequence of each enzyme is a direct translation of the nucleotide sequence in a segment of the genetic material. It appeared to be more reasonable, therefore, to consider *P. rettgeri* as a non-proteolytic and mannitol-fermenting variant of *P. vulgaris*.

Proteus morganii has characteristics which are quite different from those of *P. vulgaris* and *P. mirabilis*; it resembles Salmonella in many respects (Morgan, 1906). *P. morganii* was later transferred to the genus *Proteus* because of its urease reaction. The study of Falkow *et al.* (1962) indicated that the G + C ratio of the DNA of *P. morganii* was similar to those of the Escherichia–Shigella–Salmonella group, and not with those of *P. vulgaris* or *P. mirabilis*. The serological non-identity of the urease of *P. morganii* with the ureases of the *P. vulgaris* group

is evidence that this species is distinct from the other *Proteus* species. Whether *P. morganii* is actually closely related to Shigella–Salmonella group remains to be proven. If it could be shown, however, that the enzymes of *P. morganii* are indistinguishable serologically from those of other enteric bacilli it would then be more reasonable to consider this organism as a urease-positive variant of these enteric bacilli. In this case, only one species of *Proteus*, namely *P. vulgaris*, should be recognized with *P. mirabilis* and *P. rettgeri* as synonyms.

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REFERENCES

BATES, J. L. & LIU, P. V. (1963). Complementation of lecithinase activities by closely related pseudomonads; its taxonomic implication. *J. Bact.* **86**, 585.

ESSELMANN, M. T. & LIU, P. V. (1961). Lecithinase production by gram-negative bacteria. *J. Bact.* **81**, 939.

FALKOW, S., RYMAN, I. R. & WASHINGTON, O. (1962). Deoxyribonucleic acid base composition of Proteus and Providence organisms. *J. Bact.* **83**, 1318.

GOODMAN, M., WOLFE, H. R. & NORTON, S. (1951). Precipitin production in chickens. IV. The effect of varying concentrations of NaCl on precipitate formation. *J. Immun.* **66**, 225.

KIRK, J. S. & SUMNER, J. B. (1931). Antiurease. *J. biol. Chem.* **94**, 21.

KIRK, J. S. & SUMNER, J. B. (1934). The reaction between crystalline urease and antiurease. *J. Immun.* **26**, 495.

LIU, P. V. (1961*a*). Identification of pathogenic pseudomonads by extracellular antigens. *J. Bact.* **81**, 28.

LIU, P. V. (1961*b*). Observations on the specificities of extracellular antigens of the genera *Aeromonas* and *Serratia*. *J. gen. Microbiol.* **24**, 145.

MEYER, K. & CHAFFEE, E. (1941). The mucopolysaccharides of skin. *J. biol. Chem.* **138**, 491.

MORGAN, H. DER. (1906). Upon the bacteriology of the summer diarrhoea in infants. *Brit. med. J.* **1**, 908.

RUSTIGIAN, R. & STUART, C. A. (1945). Biochemical and serological relationships of organisms of genus *Proteus*. *J. Bact.* **49**, 419.

SUMNER, J. B. (1926). The isolation and crystallization of the enzyme urease. *J. biol. Chem.* **69**, 435.

SUMNER, J. B. (1928). Crystalline urease. II. *J. biol. Chem.* **76**, 149.

SUSKIND, S. R., YANOFSKY, C. & BONNER, D. M. (1955). Allelic strains of Neurospora lacking tryptophan synthetase; a preliminary immunochemical characterization. *Proc. natn. Acad. Sci., U.S.A.* **41**, 577.

VIEU, J. F. (1958). Note préliminaire sur la lysotypie de *Proteus hauseri*. *Z. Bakt. I. Abt. Orig.*, **171**, 612.

WENNER, J. J. & RETTGER, L. F. (1919). A systematic study of the Proteus group of bacteria. *J. Bact.* **4**, 331.

YANOFSKY, C. (1960). The tryptophan synthetase system. *Bact. Rev.* **24**, 221.

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Plaque Formation by Vaccinia Virus in Tissue Cultures Inhibited by 5-Fluorodeoxyuridine

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SUMMARY

Vaccinia virus forms plaques in monolayers of RK 13, HEp.2 and HeLa cells grown in concentrations of 5-fluorodeoxyuridine (FUDR) higher than those previously claimed to inhibit completely the synthesis of vaccinia virus DNA in suspended tissue cultures. The limited virus synthesis observed is not due to a varying susceptibility of the cells to FUDR, nor to the selection of virus resistant to FUDR. Experiments suggest that virus synthesis is made possible by thymidine from the DNA of dead cells.

INTRODUCTION

In many tissues 5-fluorodeoxyuridine (FUDR) blocks the conversion of deoxyuridylic acid to thymidylic acid, thus inhibiting DNA synthesis (Cohen *et al.* 1958). FUDR is only effective in the absence of thymidine, the latter compound annulling the inhibition (Shatkin & Salzman, 1963). The inhibitory action of FUDR has been used in studies on the synthesis of vaccinia virus in tissue culture. It has been claimed that a concentration of μM-FUDR completely inhibits vaccinia virus synthesis in suspended HeLa cells (Salzman, Shatkin & Sebring, 1963), and HEp.2 cells (Easterbrook, 1963). The observations made were, first, that cells infected with vaccinia virus and grown in the presence of FUDR had a virus titre after incubation for 24 hr which was less than the inoculum (Salzman *et al.* 1963; Easterbrook, 1963); secondly, it was shown by Easterbrook that infected suspended HEp.2 cells did not incorporate radio-active DNA precursors in the presence of FUDR. However, Gomatos *et al.* (1962), using monolayers of L cells treated with μM-FUDR obtained a vaccinia virus titre which was 12% of the uninhibited control after 24 hr incubation. Recently we have observed plaque formation by vaccinia virus in monolayers of RK 13, HEp.2 and HeLa cells in the presence of high concentrations of FUDR. With a moderate inoculum plaque formation indicates localized virus production, and implies that virus DNA synthesis must have occurred. Such synthesis could occur if some cells in a monolayer were less susceptible to FUDR than the rest, thus supporting limited virus growth. Again, some virus particles might be more capable of replication in the presence of FUDR than others. Loddo, Schivo & Ferrari (1963) showed that vaccinia virus resistant to the related inhibitor 5-iododeoxyuridine could be developed by serial passage through human amnion cells treated with the compound. A third possibility is that localized virus synthesis, and hence plaque formation, could occur if FUDR inhibition was annulled by thymidine at discrete loci on the cell sheet. Synthesis of a DNA virus, equine

abortion virus, in FUDR-treated L cells has been recently shown to take place by the use of thymidine from the DNA of damaged cells (Gentry, Lawson & Randall, 1964). The results given in the present paper suggest that this last possibility is the mechanism which permits limited vaccinia virus synthesis in cells inhibited by FUDR.

METHODS

Virus. The Lister Institute strain of vaccinia virus was used throughout, and until this time had been maintained solely on the rabbit dermis.

Cell lines. Three cell lines were used: the RK 13 line of transformed rabbit kidney cells (McCarthy, Taylor-Robinson & Pillinger, 1963), the HEp.2 line of human carcinoma cells, and the HeLa line of human carcinoma cells. The last cells were provided by Dr Bruce White of Liverpool Public Health Laboratory. RK 13 and HEp.2 cells were grown as monolayers in Parker's 199 medium containing 5% (v/v) calf serum. HeLa cells had 5% (v/v) human serum added. Incubation was at 35° in an atmosphere of 5% (v/v) CO_2 in air.

5-fluorodeoxyuridine. FUDR (the gift of Roche Products Ltd., Welwyn Garden City, Hertfordshire) was kept as a 0·1 M-solution at −20° and diluted to the required concentration in Eagle's tissue culture medium when needed.

Growth curves. Confluent monolayers of RK 13 cells were grown for 22 hr in 2% (v/v) calf serum Eagle's medium (CSE) containing 2 μM-FUDR. Eagle's medium lacks thymidine, and this treatment depleted endogenous thymidine. The medium was removed and 10^4 pock-forming units (p.f.u.) vaccinia virus allowed to absorb to each monolayer for 1 hr at 37°. The monolayers were washed with CSE medium and divided into six series. One series was set aside for titration of the virus present 1 hr after addition of the virus. CSE medium was added to one series and CSE medium containing 1, 3, 10 and 50 μM-FUDR added to the others. Groups of monolayers from control and inhibited series were then sampled at 4, 8, 12, 24 and 48 hr after addition of virus. The medium was removed, the cells disrupted ultrasonically and titrated for vaccinia on the chorioallantois (CAM) of 12-day chick embryos by the method of McCarthy & Dumbell (1961). The titres obtained by pock counts on the CAM were the same as those obtained by plaque counts on RK 13 monolayers.

Plaque inhibition tests. These were done on RK 13, HEp.2 and HeLa cell monolayers. Before inoculation the monolayers were treated as described above. 240 p.f.u. vaccinia virus were allowed to absorb to each monolayer for 1 hr at 37°, after which time the inoculum was removed and CSE medium containing FUDR added. Duplicate sets of dishes were stained with carbol fuchsin at 24 and 48 hr, and the plaques counted. Plaque diameters were measured at 48 hr. An agar overlay was unnecessary because at the time the plaques were counted, 24 hr, secondary plaque production was negligible. The plaques in RK 13 monolayers inhibited by 50 μM-FUDR were counted at 48 hr, but it is known that secondary plaque formation by vaccinia virus in these cells is not apparent until after this time (C. R. Madeley, personal communication).

Susceptibility of RK 13 cells to FUDR. RK 13 cells were trypsinized and distributed as monodisperse suspensions in CSE medium to 2 in. Petri dishes. Similar cells were suspended in 2 μM-FUDR in CSE medium. The dishes were incubated and examined daily for signs of cell division.

Virus resistance to FUDR. High-titre vaccinia virus was passaged once through RK 13 cells grown in 10 μM-FUDR in CSE medium. The virus was harvested at 24 hr and its plaquing ability in RK 13 cells in 10 and 20 μM-FUDR compared with that of the original virus stock.

The effect of dead cells on plaque formation in FUDR-treated RK 13 cells. RK 13 cells which had been grown for 22 hr in 2 μM-FUDR + CSE medium were killed by one of the following methods; heating to 56° for 3 min., freezing and thawing, ultrasonic disruption, and ultraviolet irradiation for 30 min. Monolayers of RK 13 cells were then infected with vaccinia in 10 μM-FUDR + CSE medium and after 1 hr various numbers of dead cells were added to the monolayers. The plaques were counted at 24 hr and measured at 48 hr.

RESULTS

Growth. The growth obtained in the presence of various amounts of FUDR are shown in Table 1. This shows that with 3 μM-FUDR and higher concentrations, the 24 hr titres approached that found at 1 hr, and were significantly higher than the titre detected during eclipse. Even after incubation for 48 hr the titres were not significantly higher than the inoculum.

Table 1. *The effect of 5-fluorodeoxyuridine (FUDR) on the synthesis of vaccinia virus in RK 13 cells*

Groups of monolayers, each infected with 10^4 p.f.u. vaccinia, were grown in the presence of various amounts of FUDR, harvested and titrated for vaccinia at the times stated.

	FUDR concentration (μM)				
	0	1	3	10	50
Incubation time (hr)	Virus titre/monolayer (p.f.u. $\times 10^{-3}$)				
1	3·5	3·5	3·5	3·5	3·5
4	0·19	0·24	0·41	0·13	0·16
8	0·39	0·21	0·72	0·17	0·37
12	43	6·7	1·8	1·3	0·68
24	800	40	3·8	3·2	2·5
48	5000	350	4·7	3·9	3·2

Plaque inhibition tests. In most cases plaques were seen and counted at 24 hr, but it was found more convenient to count the very small plaques produced in RK 13 cells in the presence of 50 μM-FUDR at 48 hr, by which time the plaques had reached maximum size. Plaque counts are shown in Table 2. It is evident that the plaque number decreased with increasing FUDR concentrations; the plaque diameters also decreased. Uninhibited plaques in RK 13 cells were 1·2 mm. diameter; in 50 μM-FUDR they were 0·2 mm. Uninhibited plaques in the HeLa cells were 0·46 mm. diameter; in 5 μM-FUDR they were 0·22 mm. In HEp.2 cells, vaccinia virus produced clumps of deeply staining cells and it was not possible to detect any size variation with different FUDR concentrations. Clearly in HEp.2 and HeLa cells plaque formation occurred in FUDR concentrations higher than those previously claimed to inhibit completely vaccinia virus DNA synthesis. The results indicate that some DNA synthesis must have occurred. Therefore, the

hypothesis suggested by Easterbrook (1963), that the recoating of inoculum virus DNA with newly synthesized protein accounts for the slight rise in titre over the eclipse level, cannot be the sole factor involved.

Susceptibility of RK 13 cells to FUDR. Monodisperse cells seeded in CSE medium settled and became attached to the glass within 24 hr, and by 48 hr they were dividing. When 2 μM-FUDR was added to the CSE medium, very few cells were attached by 48 hr, and none was seen to divide. This suggests that 2 μM-FUDR is sufficient to inhibit DNA replication in all RK 13 cells, and that no cells remain unaffected.

Table 2. *Plaque production by vaccinia virus in monolayers of RK 13, HEp.2 and HeLa cells treated with 5-fluorodeoxyuridine (FUDR)*

A control count of 240 plaques/monolayer was attempted. In this Table control counts have been reduced to 100, and all other counts adjusted accordingly. Plaques were counted at 24 hr; except that plaques in RK 13 cells treated with 50 μM-FUDR were counted at 48 hr.

	FUDR concentration (μM)				
Cell line	0	1	5	10	50
RK 13	100	35	24	11	8
HEp.2	100	49	25	8	N.T.*
HeLa	100	17	4	†	N.T.

* N.T. = not tested; † Unequivocal demonstration of plaques not possible.

Virus resistance to FUDR. There was no increased resistance to FUDR in vaccinia which had been passaged once in the presence of 10 μM-FUDR. Plaque production by such virus was decreased by 91% in 10 μM and by 96% in 20 μM-FUDR. That of the original virus was decreased by 88% in 10 μM and 91% in 20 μM-FUDR. Hence it is unlikely that vaccinia virus synthesis in FUDR-treated cells was initiated by virus resistant to FUDR.

The effect of dead cells on plaque formation in FUDR-treated RK 13 cells. Trypan Blue staining showed that about 1·2% of the RK 13 cells were dead after 22 hr in 2 μM-FUDR + CSE medium. Such monolayers infected with 160 p.f.u. vaccinia in 10 μM-FUDR developed 14 plaques of 0·4 mm. maximum diameter. Thymidine-depleted dead cells were added to similar monolayers and when the number of dead cells added was the same as the number of dead cells originally present, there were 25 plaques of maximum diameter 0·45 mm. When ten times the original number of dead cells were added there were 53 plaques of maximum diameter 0·56 mm. These results suggest that thymidine from the DNA of dead cells was used for vaccinia virus synthesis, and that there were sufficient dead cells in a monolayer to support the limited virus synthesis observed in high concentrations of FUDR. Shatkin & Salzman (1963) suggested that any thymidine available will be used preferentially for virus synthesis.

DISCUSSION

Examination of Tables 1 and 2 shows an apparent discrepancy between the results obtained by the two methods used, namely, total virus counts and plaque counts. In 10 μM-FUDR, for example, only 0·1% virus synthesis was indicated by

the growth curve, whereas the number of plaques produced was 11 % as compared to the control. However, the latter estimate is of plaque number only and does not take into account the smaller area of necrosis, and the smaller virus yield/cell. The area of a plaque in 10 μM-FUDR was 0·12 sq. mm. as compared to 1·1 sq. mm. for the control; hence the discrepancy was decreased from a 100-fold to 10-fold. This suggests that 10 μM-FUDR decreased the virus yield/cell to about 10 % of that of the control, but that in spite of the lower yield of virus, cell necrosis was found in inhibited cultures. A similar relationship was shown for other concentrations of FUDR. The results presented indicate that complete inhibition of vaccinia virus synthesis by FUDR is difficult, if not impossible, to obtain in monolayer culture. Dead cells in such cultures may act as sources of thymidine which may permit the formation of plaques. It is possible that complete inhibition may be easier to achieve in suspended cultures since any thymidine present will be rapidly dispersed and diluted. It appears that virus synthesis in the presence of FUDR may not be limited to vaccinia virus. The synthesis of bovine abortion virus by the mechanism suggested above has already been mentioned (Gentry *et al.* 1964). Lam & Atherton (1963) only found 93 % plaque inhibition of herpes simplex virus in L cells inhibited by 80 μM-FUDR. Undoubtedly FUDR markedly decreases the titre of vaccinia virus in tissue culture. In view of the results presented here, however, care must be taken when interpreting experiments on viral protein synthesis in FUDR-treated tissue cultures (Salzman *et al.* 1963), since complete inhibition of infective virus may not have been achieved.

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REFERENCES

COHEN, S. S., FLAKS, J. S., BARNER, H. D., LOEB, M. R. & LICHTENSTEIN, J. (1958). The mode of action of 5-fluorouracil and its derivatives. *Proc. natn. Acad. Sci., Wash.* **44**, 1004.

EASTERBROOK, K. B. (1963). Conservation of vaccinial DNA during an abortive cycle of multiplication. *Virology*, **21**, 508.

GENTRY, G. A., LAWSON, L. & RANDALL, C. C. (1964). Replication of a DNA virus in the presence of an inhibitor of DNA synthesis. *Fed. Proc.* **23**, 131.

GOMATOS, P. J., TAMM, I., DALES, S. & FRANKLIN, R. M. (1962). Reovirus type 3: physical characteristics and interaction with L cells. *Virology*, **17**, 441.

LAM, K. S. K. & ATHERTON, J. G. (1963). Measles virus. *Nature, Lond.* **197**, 820.

LODDO, B., SCHIVO, M. L. & FERRARI, W. (1963). Development of vaccinia virus resistant to 5-iodo-2-deoxyuridine. *Lancet*, ii, 914.

MCCARTHY, K. & DUMBELL, K. R. (1961). Chorioallantoic inoculation of eggs—improved method. *Virology*, **14**, 488.

MCCARTHY, K., TAYLOR-ROBINSON, C. H. & PILLINGER, S. (1963). Isolation of rubella virus from cases in Great Britain. *Lancet*, ii, 593.

SALZMAN, N. P., SHATKIN, A. J. & SEBRING, E. D. (1963). Viral protein and DNA synthesis in vaccinia virus-infected HeLa cell cultures. *Virology*, **19**, 542.

SHATKIN, A. J. & SALZMAN, N. P. (1963). Deoxyribonucleic acid synthesis in vaccinia virus-infected HeLa cells. *Virology*, **19**, 551.

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The Effect of *p*-Fluorophenylalanine on the Replication of Rabbitpox Virus and its Nucleic Acid

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SUMMARY

The multiplication of rabbitpox virus in HeLa (ERK) cells was inhibited by *p*-fluorophenylalanine (FPA) 400 μg./ml. When added early in the growth cycle, FPA prevented the formation of viral DNA. The FPA-sensitive process necessary for DNA synthesis began about $1\frac{1}{4}$ hr after infection. This was at least 1 hr before the first development of viral DNA, but coincided with the time of synthesis of the earliest viral antigens. On the basis of this and other evidence, it is suggested that some soluble viral antigens are enzymes concerned in the synthesis of viral DNA.

INTRODUCTION

When rabbitpox virus multiplies in HeLa (ERK) cells, virus maturation is preceded by the synthesis of viral DNA and of viral antigens (Appleyard & Westwood, 1964). DNA synthesis begins $2\frac{1}{2}$ hr after infection, which is $2\frac{1}{2}$ hr before the first appearance of new virus. The numerous soluble antigens are formed at various stages of growth; some of them cannot be detected until after the start of virus maturation, whereas others appear only 1–$1\frac{1}{2}$ hr after infection. The synthesis of viral antigens is independent of the synthesis of viral DNA, for not only are some antigens formed before DNA, but the development of all of them is unaffected by bromodeoxyuridine. In the present work we have used the compound *p*-fluorophenylalanine, which inhibits the formation of infective virus, to show that the formation of viral DNA, on the other hand, does depend upon the synthesis of protein. The time of the essential protein synthesis was measured, and related to the time of appearance of soluble viral antigens.

METHODS

The techniques used were similar to those of Appleyard & Westwood (1964) and may be summarized briefly.

Virus. This was the Utrecht strain of rabbitpox virus, adapted to HeLa (ERK) cells.

Cell cultures. HeLa (ERK) cultures were normally used as confluent monolayers in 8 cm. Carrel flasks. When cells were to be stained with acridine orange, semi-confluent monolayers were grown on coverslips.

Virus growth. Cell cultures in Carrel flasks were incubated for 1 hr at 36° with 2 ml. rabbitpox virus suspension of titre 10^8 plaque-forming units (p.f.u.)/ml.; this

resulted in infection of over 90% of the cells. Unadsorbed virus was removed by washing with phosphate buffered saline, then 10 ml. maintenance medium added and the incubation continued for periods up to 24 hr. All growth periods were measured from the time at which cultures were first inoculated with virus.

Virus titration. Infected cultures were harvested by scraping the cells from the glass and disrupting them by ultrasonic treatment. The virus content was titrated as plaque-forming units (p.f.u.) in HeLa (ERK) monolayers.

Detection of viral antigens. Cultures to be tested for soluble viral antigens were first concentrated to 4×10^7 cells/ml. and then disrupted ultrasonically. The resulting extract was examined by micro-immunodiffusion against hyperimmune rabbitpox serum.

Detection of viral DNA. Cell cultures on coverslips were infected by incubation for 1 hr at 36° with 0·2 ml. rabbitpox virus of titre 10^8 p.f.u./ml. After washing, incubation was continued in maintenance medium. At different stages of virus growth, sample cultures were fixed in ethanol, stained with acridine orange, and examined under dark-ground ultraviolet illumination for green cytoplasmic inclusions.

RESULTS

Effect of p-fluorophenylalanine on virus multiplication

DL-*p*-fluorophenylalanine (FPA), at a concentration of 400 μg./ml. or above, completely inhibited the formation of infective virus (Fig. 1). FPA interferes with protein synthesis, either by preventing it or by becoming incorporated into protein in the place of phenylalanine (Cohen & Munier, 1959). The synthesis of a great many proteins is likely to be necessary for the multiplication of rabbitpox virus, and it would therefore be expected that several stages of the growth cycle would be sensitive to inhibition by FPA.

To estimate the time of the last FPA-sensitive stage of growth, FPA 400 μg./ml. was added to a series of cultures at various times after infection, and the final virus yields titrated after incubation for 24 hr. FPA completely prevented virus replication when added as late as 3 hr after infection, but a small amount of virus was produced when it was not added until 4 hr. Since, during normal growth, new virus first appeared 5 hr after infection, it follows that the last FPA-sensitive stage of growth occurred about 1 hr before virus maturation. This conclusion was confirmed by studying the course of virus growth in cultures to which FPA at concentrations of 400, 800 or 1600 μg./ml. was added 6 hr after infection. Virus increase continued for 1 hr after the addition of each of these concentrations of inhibitor, and then ceased abruptly.

The inhibition caused by FPA was annulled by its removal and replacement with normal medium. This made it possible to time the earliest stage at which FPA acted on virus growth. One-step growth experiments were made in normal cell cultures and in cultures to which FPA 400 μg./ml. was added for the first 2 hr or 4 hr after infection (Fig. 2). The exposure of cultures to FPA for 2 hr delayed growth by only about ½ hr and exposure for 4 hr caused a delay of about 3 hr. The first FPA-sensitive stage in virus growth therefore began 1–1½ hr after infection.

Effect on the synthesis of viral antigens

Infected cultures were incubated for 24 hr either in normal medium or in medium containing FPA at concentrations up to 1600 μg./ml. The yields of virus from these cultures are shown by the triangles in Fig. 1. Examination of cell extracts by immunodiffusion showed that the lowest concentration of FPA that completely inhibited infective virus production (400 μg./ml.) had only a very slight effect on the synthesis of soluble antigens. Higher concentrations did prevent the formation of

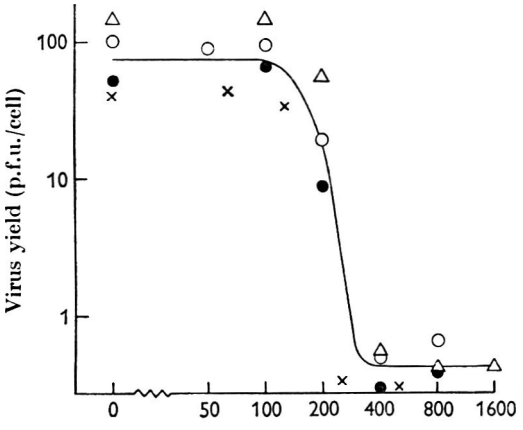

Fig. 1

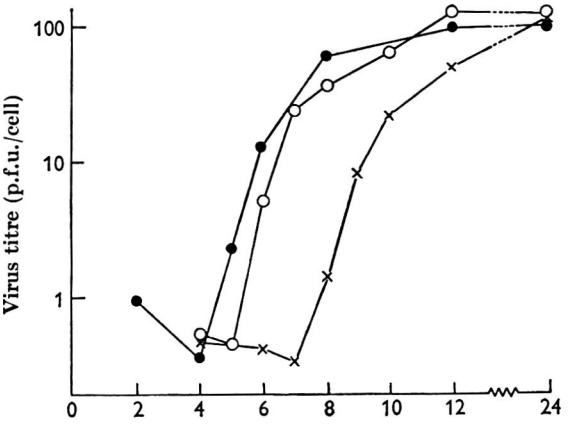

Fig. 2

Fig. 1. Effect of *p*-fluorophenylalanine (FPA) on the 24 hr yield of rabbitpox virus from HeLa (ERK) cultures. The symbols represent the results of four different experiments.

Fig. 2. Growth of rabbitpox virus after temporary exposure of infected cultures to FPA. ●, normal growth; ○, growth after exposure to FPA for first 2 hr after infection; ×, growth after exposure to FPA for first 4 hr after infection.

many antigens, but at least six antigens were produced even in the presence of FPA 1600 μg./ml.; the yield of a few antigens was increased by FPA at concentrations of 400 μg./ml. and above. Since FPA is known to inhibit a stage of growth that occurs within 1½ hr of infection, its effect on the synthesis of the early viral antigens was tested. Virus was grown for only 2 hr in the presence or absence of FPA 400 μg./ml., and the cell extracts were then examined by immunodiffusion. Identical patterns of nine precipitin lines were produced by the antigens from both control and inhibited cultures.

Effect on the formation of viral nucleic acid

Experiments with acridine orange. During normal virus growth (Appleyard & Westwood, 1964), the staining of cultures with acridine orange showed multiple small foci of DNA in the cytoplasm of a few cells 2½ hr after infection, and within another 2 hr almost all the cells developed DNA. The foci steadily increased in size, began to spread and fragment about 6 hr after infection, and by 24 hr DNA was diffused widely through the cytoplasm. To investigate the effect of FPA on the formation of viral DNA, infected cell cultures on coverslips were incubated in

medium with or without FPA 400 μg./ml. Sample cultures were stained with acridine orange at 5, 8 and 24 hr. The control cultures showed the normal development of viral DNA. But the cells of inhibited cultures contained no cytoplasmic DNA at 5 hr or 8 hr and only a few very small localized foci at 24 hr. Other experiments gave similar results, except that the cells of inhibited cultures sometimes showed a few minute DNA inclusions at 8 hr.

The effect of adding FPA at different times in the virus growth cycle was then studied. A series of cultures on coverslips was infected in the usual way, and FPA 400 μg./ml. was added either with the virus or at various times up to 4 hr after infection. Cultures were stained with acridine orange 7 hr after infection, the cells containing DNA were counted and the appearance of the foci noted. In addition, the course of DNA development was followed at hourly intervals in uninhibited cultures and in cultures to which FPA was added at 2 hr (Fig. 3). It was found that the addition of FPA either with the virus or 1 hr after infection almost completely suppressed DNA formation, the cells that did contain DNA at 7 hr having only a

Table 1. *Inhibition of the growth of rabbitpox virus by p-fluorophenylalanine (FPA) followed by bromodeoxyuridine (BDU)*

Treatment of culture	Virus yield at 24 hr (p.f.u./cell)
None	87
BDU (20 μg./ml.) 8–24 hr	73
FPA (400 μg./ml.) 0–8 hr	124
FPA 0–8 hr+BDU 8–24 hr	0·42

few very small inclusions. But FPA added 2 hr after the virus only partially inhibited DNA synthesis, and when added at 3 hr or 4 hr it was even less effective. These results indicated that an FPA-sensitive process was essential for the synthesis of viral DNA, and that this process began between 1 hr and 2 hr after virus infection.

Experiments with bromodeoxyuridine. The multiplication of rabbitpox virus was inhibited by bromodeoxyuridine (BDU) 20 μg./ml. (Appleyard & Westwood, 1964). Inhibition was caused by the incorporation into viral DNA of BDU in the place of thymidine, thus producing non-functional nucleic acid. The course of viral DNA synthesis could be studied by measuring the final yields of virus from a series of cultures to which BDU had been added at different times after infection. Under normal circumstances, BDU caused little decrease in the final virus yield when added 8 hr after infection of a culture, for almost all the viral DNA had already been synthesized by this time. However, when FPA was included in the medium for the first 8 hr, then the addition of BDU at this time resulted in complete inhibition of virus growth (Table 1). Temporary exposure to FPA did not itself decrease the virus yield; on the contrary there was a slight increase, which was frequently observed when FPA was added to cultures at the time of infection and later removed.

This result showed that FPA prevented the synthesis of viral DNA. In order to time the FPA-sensitive step in DNA synthesis, the same type of experiment was repeated except that FPA was added to cultures at progressively later times after infection. The final virus yields obtained when FPA was added at different times and then replaced at 8 hr with medium containing BDU are shown in Fig. 4, curve

C. For comparison, curve A of this figure is a normal virus growth curve, and curve B shows the course of viral DNA synthesis obtained by titrating the 24 hr yields of virus after addition of BDU at the times indicated. It can be seen from curve C that FPA completely prevented DNA synthesis when added either with the virus or 1 hr later, but by 2 hr after infection the cultures were beginning to pass the sensitive stage of growth. The unexpectedly low yield of virus from the cultures to which FPA was added at 4 hr or later was a usual occurrence. Temporary exposure of cultures to FPA between 4 hr and 8 hr after infection decreased the virus yield even without subsequent treatment with BDU.

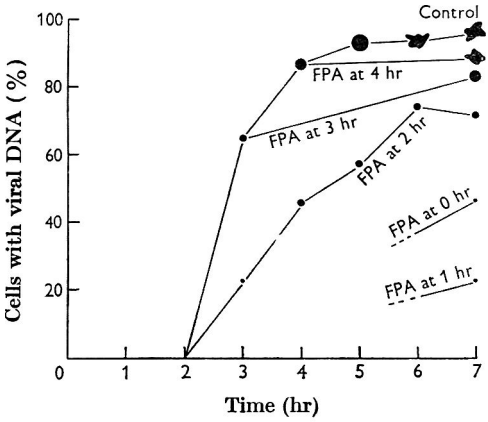

Fig. 3

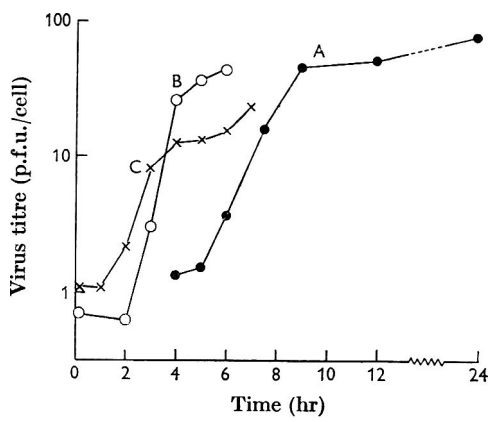

Fig. 4

Fig. 3. Effect of time of addition of FPA on its ability to inhibit the formation of DNA inclusions by infected cultures. The % cells containing inclusions were counted at the times shown. The size of each symbol is proportional to that of the typical inclusions in a particular culture, and its shape indicates whether the inclusions were compact or spreading.

Fig. 4. Effect of time of addition of FPA on its ability to inhibit the synthesis of viral DNA by infected cultures. ●, virus growth curve; ○, curve of DNA formation, obtained by titrating the virus yield at 24 hr from cultures to which bromodeoxyuridine (BDU) was added at the times shown; ×, virus yield at 24 hr after addition of FPA at times shown followed at 8 hr by removal of FPA and addition of BDU.

Fig. 5 shows the results of a similar experiment designed to determine more precisely the time at which the FPA-sensitive process began. The three curves were obtained as before, but observations were made more frequently and confined to the early period of growth. If a level of 1 p.f.u./cell is arbitrarily taken as the first significant rise in titre, then virus maturation began at about 5 hr, DNA formation at about 2 hr 40 min., and the FPA-sensitive reaction at about 1 hr 15 min.

The start of the FPA-sensitive step in DNA synthesis could also be timed by measuring the delay in DNA synthesis caused by the temporary exposure of infected cultures to FPA. In the experiment illustrated in Fig. 6, four groups of cultures were infected with rabbitpox virus. The control group was incubated without FPA, whereas the other three were treated with FPA 400 μg./ml. for the first 1, 2 or 3 hr of incubation. DNA formation was followed during the early stages of virus growth by titrating the final virus yields from cultures to which BDU was added at different times. When, as before, a value of 1 virus equivalent/cell is taken as the first

significant increase in DNA, then DNA synthesis began at about 2 hr 35 min. in the control cultures. Synthesis was unaffected by the presence of FPA for the first 1 hr after infection; but exposure for 2 hr or 3 hr delayed the first appearance of DNA until about 3 hr 25 min or 4 hr 20 min., respectively. This indicated that the FPA-sensitive step began about 1 hr 15 min. after infection.

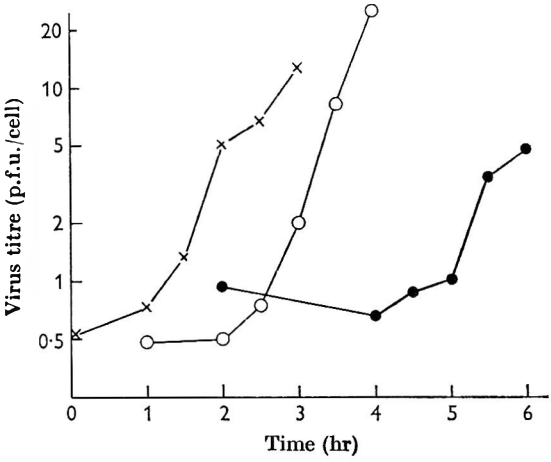

Fig. 5

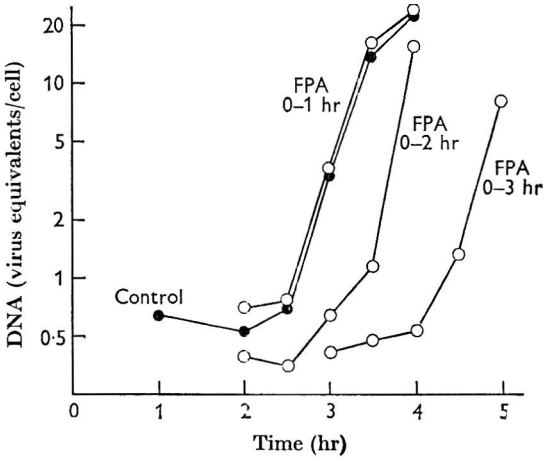

Fig. 6

Fig. 5. Effect of time of addition of FPA on its ability to inhibit the synthesis of viral DNA by infected cultures. The symbols ●, ○ and × have the same meanings as in Fig. 4.

Fig. 6. Course of synthesis of viral DNA after temporary exposure of infected cultures to FPA. All points were obtained by titrating the virus yield at 24 hr from cultures to which BDU was added at the times shown. ●, normal cultures; ○, cultures exposed to FPA for the first 1, 2 or 3 hr after infection.

DISCUSSION

The ability of *p*-fluorophenylalanine (FPA) to inhibit viral DNA synthesis showed that the formation of new protein was essential for the development of viral DNA. Various experiments designed to time the necessary protein synthesis gave consistent results, and indicated that the process began about $1\frac{1}{4}$ hr after infection. In the interpretation of these experiments it was assumed that FPA began to act without appreciable delay when added to a culture and ceased to act as soon as it was removed. The assumption probably caused little error, for it is known that amino acids in the medium equilibrate rapidly with the amino acid pool of cells in culture (Eagle, 1959). Also, Wecker & Schonne (1961) found that FPA was incorporated without detectable lag into the proteins of cultured chick embryo cells, and that incorporation ceased as soon as phenylalanine was added to the medium.

The effect of FPA on DNA synthesis was not due to interference with the initiation of infection, for even high concentrations of FPA allowed the formation of several soluble viral antigens. It is known that the growth of vaccinia virus causes increased activity of at least two enzymes (thymidine kinase, DNA polymerase), which is first detectable in cells about 2 hr after infection (Green & Pina, 1962;

Magee, 1962; McAuslan & Joklik, 1962; Kit, Piekarski & Dubbs, 1963), and that the increase can be prevented by FPA or puromycin (McAuslan & Joklik, 1962; Kit, Dubbs & Piekarski, 1963). It is therefore likely that the inhibition of DNA synthesis by FPA in our experiments was due to interference with the formation of these or similar enzymes. Normal cell enzymes can participate in the formation of viral DNA, for Dubbs & Kit (1964) found that a mutant of vaccinia, which was unable to induce thymidine kinase, incorporated tritiated thymidine into cytoplasmic foci when growing in L cells that did contain the enzyme. It is now apparent that the pre-existing cell enzymes on their own are unable to complete the synthesis of any viral DNA.

New enzymes might be formed under the influence of viral DNA or they might be cellular enzymes whose synthesis had been stimulated by infection. Recent evidence suggests the former possibility. Kit, Piekarski & Dubbs (1963) showed that a subline of L cells lacking thymidine kinase nevertheless formed this enzyme when infected with vaccinia virus, and McAuslan (1963) found that the thymidine kinase induced by infection of HeLa cells with cowpox virus differed in heat stability and K_m value from the enzyme of uninfected cells.

If the new enzymes were formed under the influence of viral DNA, they would have the antigenic specificity of virus rather than of host cell. Westwood, Zwartouw, Appleyard & Titmuss (1965) found that ten of the soluble viral antigens formed during the growth of vaccinia virus were apparently not present in the virus particles, and suggested that these antigens might be enzymes concerned in virus synthesis. The first soluble antigens formed during the growth of rabbitpox virus in HeLa (ERK) cells appear between 1 and $1\frac{1}{2}$ hr after infection (Appleyard & Westwood, 1964). The present estimate of $1\frac{1}{4}$ hr after infection for the start of the FPA-sensitive step in viral DNA synthesis agrees very well with this time. This suggests that some of the soluble viral antigens, particularly those formed early in virus growth, are enzymes required for viral DNA synthesis. If this conclusion be correct, it might be expected that FPA, at a concentration sufficient to prevent DNA synthesis, would also inhibit synthesis of the early viral antigens. The fact that this did not occur may be explained by the formation of altered proteins that, although non-functional due to their content of FPA, were antigenically indistinguishable from the active enzymes produced during normal virus growth.

We wish to thank Miss H. J. Way for her skilled technical assistance throughout this work.

REFERENCES

Appleyard, G. & Westwood, J. C. N. (1964). The growth of rabbitpox virus in tissue culture. *J. gen. Microbiol.* **37**, 391.

Cohen, G. N. & Munier, R. (1959). Effets des analogues structuraux d'aminoacides sur la croissance, la synthèse de protéines et la synthèse d'enzymes chez *Escherichia coli*. *Biochim. biophys. Acta*, **31**, 347.

Dubbs, D. R. & Kit, S. (1964). Isolation and properties of vaccinia mutants deficient in thymidine kinase-inducing activity. *Virology*, **22**, 214.

Eagle, H. (1959). Amino acid metabolism in mammalian cell cultures. *Science*, **130**, 432.

Green, M. & Pina, M. (1962). Stimulation of the DNA-synthesizing enzymes of cultured human cells by vaccinia virus infection. *Virology*, **17**, 603.

KIT, S., DUBBS, D. R. & PIEKARSKI, L. J. (1963). Inhibitory effects of puromycin and fluorophenylalanine on induction of thymidine kinase by vaccinia-infected L cells. *Biochem. biophys. Res. Comm.* **11**, 176.

KIT, S., PIEKARSKI, L. J. & DUBBS, D. R. (1963). Induction of thymidine kinase by vaccinia-infected mouse fibroblasts. *J. molec. Biol.* **6**, 22.

MCAUSLAN, B. R. (1963). The induction and repression of thymidine kinase in the pox-virus-infected HeLa cell. *Virology*, **21**, 383.

MCAUSLAN, B. R. & JOKLIK, W. K. (1962). Stimulation of the thymidine phosphorylating system in HeLa cells on infection with pox virus. *Biochem. biophys. Res. Comm.* **8**, 486.

MAGEE, W. E. (1962). DNA polymerase and deoxyribonucleotide kinase activities in cells infected with vaccinia virus. *Virology*, **17**, 604.

WECKER, E. & SCHONNE, E. (1961). Inhibition of viral RNA synthesis by parafluorophenylalanine. *Proc. natn. Acad. Sci., U.S.A.* **47**, 278.

WESTWOOD, J. C. N., ZWARTOUW, H. T., APPLEYARD, G. & TITMUSS, D. H. J. (1965). Comparison of the soluble antigens and virus particle antigens of vaccinia virus. *J. gen. Microbiol.* **38**, 47.

Books Received

An Introduction to Microbiology. By W. B. HUGO. Published by William Heinemann Medical Books Ltd., 23 Bedford Square, London, W.C. 1. 139 pp. Price 20*s*.

An Introduction to Parasitology. By JOHN M. WATSON. Published by William Heinemann Medical Books Ltd., 23 Bedford Square, London, W.C. 1. 180 pp. Price 20*s*.

Gene Action. By PHILIP E. HARTMAN and SIGMUND R. SUSKIND. Published by Prentice-Hall International, 28 Welbeck Street, London, W. 1. 148 pp. Price 21*s*. paper and 40*s*. cloth.

Plant Virology. Edited by M. K. CORBETT and H. D. SISLER. Published by The University of Florida Press, 15 N.W. 15th Street, Gainesville, Florida, U.S.A. 493 pp. Price $12.50.

Revista Venezolana de Sanidad y Asistencia Social. Vol. 28, no. 4, 1963. Published by Ministerio de Sanidad y Asistencia Social, Caracas, Venezuela.

Revista Venezolona de Sanidad y Asistencia Social. Vol. 29, no. 1, 1964. Published by Ministerio de Sanidad y Asistencia Social, Caracas, Venezuela.

Techniques in Experimental Virology. Edited by R. J. C. HARRIS. Published by the Academic Press Inc. (London), Ltd., Berkeley Square House, Berkeley Square, London, W.1. 425 pp. Price 100*s*.

Thermophilic Fungi. By DONALD G. COONEY and RALPH EMERSON. Published by W. H. Freeman & Co. Ltd., Warner House, 48 Upper Thames Street, London, E.C. 4. 182 pp. Price 36*s*.

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