Chlorine-containing compounds produced during *Dictyostelium* development

Detection by labelling with ^{36}Cl

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DIF-1 [Differentiation-Inducing Factor 1; 1-(3,5-dichloro-2,6-dihydroxy-4-methoxyphenyl)hexan-1-one] is a novel chlorinated signal molecule that induces stalk-cell differentiation during development of *Dictyostelium discoideum*. Here we introduce the use of the radioisotope ^{36}Cl to label DIF-1 and other low- M_r chlorinated compounds produced during development. H.p.l.c. and t.l.c. were used to resolve the labelled compounds. We find the following. (1) At least 14 dialysable ^{36}Cl -labelled compounds are released into the medium by cells labelled continuously through development with Na^{36}Cl . (2) The compounds can be classified into two major groups according to their times of accumulation in development. The early group of compounds starts accumulating at the end of aggregation, co-ordinately with DIF-1; the late group is only made at the end of development, by mature fruiting bodies. There may also be an intermediate group made during culmination. (3) The early group of compounds has been identified as comprising DIF-1 and seven of its metabolites by co-chromatography with the authentic compounds. These metabolites had previously only been recognized in suspensions of living cells incubated with exogenous DIF-1. Their detection here, from cells undergoing normal development, suggests that endogenous DIF-1 is metabolized in normal development in much the same way as is DIF-1 added to cells in suspension. (4) The intermediate and late groups of compounds are not obvious DIF-1 metabolites. They may have some role unconnected with DIF signalling. (5) A group of ^{36}Cl -labelled late compounds remain cell-associated after washing of the fruiting bodies, and these are greatly enriched in stalk, compared with spore, cells. (6) Other slime-mould species were labelled with ^{36}Cl . All three tested, namely *D. mucoroides*, *D. vinaceo-fuscum* and *P. violaceum*, also produced chloro compounds. *D. mucoroides* produced DIF-1 by the criterion of h.p.l.c. co-elution with authentic DIF-1. A developmentally regulated metabolism of chlorinated compounds may therefore be widespread amongst slime moulds. To our knowledge, labelling with ^{36}Cl *in vivo* has not been reported before and provides a powerful general method for investigating chlorinated compounds in diverse organisms.

INTRODUCTION

Development in *Dictyostelium* is regulated by signalling between the cells (Loomis, 1982; Gerisch, 1987; Williams, 1988). During early development, the initially separate amoebae aggregate together in response to cyclic AMP signals to form small mounds of cells. Within these mounds, prestalk and prespore cells differentiate and become organized into discrete tissues. The mound elongates into a standing first finger, which may fall on its side and migrate, before forming a fruiting body consisting of a cellular stalk supporting a mass of spores. Prestalk and stalk-cell differentiation is induced by a low- M_r chlorinated alkyl phenone, known as DIF-1 [Differentiation-Inducing Factor 1; 1-(3,5-dichloro-2,6-dihydroxy-4-methoxyphenyl)hexan-1-one], which is active at nanomolar concentrations (Town *et al.*, 1976; Town & Stanford, 1979; Kopachik *et al.*, 1983; Morris *et al.*, 1987; Williams *et al.*, 1987). DIF-1 is believed to be able to penetrate cell membranes, and a cytoplasmic binding protein has been detected, which may mediate its effects on gene expression (Insall & Kay, 1990). When labelled DIF-1 is added back to cells, it is metabolized to produce at least ten compounds more polar than itself (Traynor & Kay, 1991). These compounds have not yet been identified and are therefore known as DIF metabolites

(DMs) 1–10. The early steps of the DIF-metabolizing pathway have been deduced by monitoring the kinetics of metabolite production and the fate of metabolites added back to cells. The first metabolite in the pathway is largely cell-associated, whereas later ones are found largely in the medium. DIF-1 (Brookman *et al.*, 1982; Sobolewski *et al.*, 1983), its binding protein (Insall & Kay, 1990) and the ability of cells to metabolize DIF-1 (Traynor & Kay, 1991) all increase dramatically at the end of aggregation; at this time the cells evidently gain the components of a DIF-1 signalling system. Apart from DIF-1, two minor activities that induce stalk-cell differentiation have been identified: DIF-2 is the pentyl homologue, and DIF-3 the monochloro analogue, of DIF-1 (Morris *et al.*, 1988).

In order to learn more about the DIF-1 signalling system we have sought a method for labelling DIF-1 *in vivo* that would allow us to monitor DIF-1 production and breakdown in various circumstances. The presence of chlorine in DIF-1 is most unusual. Although some 200 chlorine-containing natural products have been identified, mainly from fungi and marine algae (Siuda & DeBernardis, 1973; Neidleman & Geigert, 1986), none, apart from the DIFs, have been identified from slime moulds. It was therefore conceivable that labelling of cells with ^{36}Cl could provide a specific way of labelling DIF-1 and related compounds.

Abbreviations used: DIF-1, Differentiation-Inducing Factor 1 [1-(3,5-dichloro-2,6-dihydroxy-4-methoxyphenyl)hexan-1-one]; DM(s), DIF-1 metabolite(s); CCDs, chlorinated compounds from *Dictyostelium*; in some comparative sections the following organism abbreviations are used: *D.d.*, *Dictyostelium discoideum*; *D.m.*, *Dictyostelium mucoroides*; *D.v.-f.*, *Dictyostelium vinaceo-fuscum*; *P.v.*, *Polysphondylium violaceum*.

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Fig. 1. H.p.l.c. separation of chlorine-containing compounds released during development by *D. discoideum*

(b) ^{36}Cl radioactivity and DIF activity. (a) DIF activity at 100-fold sensitivity to show the minor DIF activities. Cells of strain Ax2, plated on cellophane, were labelled with Na^{36}Cl ($0.025 \mu\text{Ci}\cdot\text{ml}^{-1}$; 0.07 mM) in DIFlab for 2 or 3 days, until development was complete. Compounds collected across the cellophane and bound by XAD-2 resin were taken for analysis (see the Materials and methods section). H.p.l.c. was performed with a 50 cm Whatman Partisil-10 M9 ODS-3 column eluted at 3 ml/min with a gradient going from 40% solvent B to 80% in 80 min and then to 100% in 20 min (solvent A = 2% acetic acid; solvent B = 2% acetic acid/98% methanol). Fractions were collected every minute, starting at 6 min, and 8% was taken for determining ^{36}Cl (these radioactivities are those given); the DIF activity was determined using the stalk-cell-differentiation bioassay and total activity/fraction is given. $\blacklozenge$, $\blacklozenge$ DIF activity; $\square$, ^{36}Cl radioactivity.

Living cells appear to incorporate chlorine into organic compounds from inorganic chloride, often by the action of a chloroperoxidase enzyme (Hager *et al.*, 1966; Neidleman &

Geigert, 1986). The only practical radioisotope of chlorine for *in vivo* labelling is ^{36}Cl , though it has the disadvantage of a very low specific radioactivity owing to its half-life of about 300 000 years. Despite the consequent low sensitivity, we have been able to use ^{36}Cl labelling to detect DIF-1 and other chlorinated compounds made during *Dictyostelium* development.

MATERIALS AND METHODS

Growth and labelling of cells

Except where indicated, *Dictyostelium discoideum*, strain Ax2 was used. These were grown in axenic medium (Watts & Ashworth, 1970) at 22°C , either in flasks shaken at 180 rev./min on a gyrotary shaker or in 20-litre stirred bubblers (Kay *et al.*, 1983). Cells were harvested at $(2-6) \times 10^6/\text{ml}$ by low-speed centrifugation and washed in KK2 (20 mM- $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4/2 \text{ mM-MgSO}_4$, pH 6.2) and finally in DIFlab (12 mM- $\text{KH}_2\text{PO}_4/8 \text{ mM-Na}_2\text{HPO}_4/1 \text{ mM-MgSO}_4$, pH 6.7) or 10% DIFlab in later experiments. To prepare the released labelled compounds, cells were allowed to develop at $7 \times 10^6/\text{cm}^2$ on thin sheets of cellophane (325P; British Cellophane, Bridgwater, Somerset, U.K.) supported by a stainless-steel mesh in a surgical tray (Kay *et al.*, 1983). The underside of the tray was bathed with DIFlab, containing $100 \mu\text{g}$ of streptomycin sulphate/ml, 5–10 g of XAD-2 resin (BDH)/litre and Na^{36}Cl (Amersham International) as indicated. At the appropriate times the XAD-2 beads were removed, washed extensively with water, and the bound compounds were eluted with several changes of 100% ethanol.

Cells of *D. discoideum*, strain V12M2, *D. mucoroides*, *D. vinaceo-fuscum* and *Polysphondylium violaceum* were grown in association with *Klebsiella aerogenes* on nutrient agar, harvested when the plates were half-cleared and freed of bacteria by differential centrifugation (Kay, 1979). To prepare cell-associated compounds, cells were allowed to develop on 1.8% agarose (BRL; electrophoresis grade) containing 10% DIFlab and

Table 1. Chromatographic characterization of the major ^{36}Cl -labelled compounds made during *Dictyostelium* development

H.p.l.c. step 1 used a 50 cm Partisil M9 ODS-3 column, eluted at 3 ml/min with gradient I: 40–80% (v/v) solvent B against solvent A in 80 min and then to 100% B in 20 min (solvent A = 2% acetic acid; solvent B = 2% acetic acid/98% methanol). H.p.l.c. step 2 used a Partisil PXS/25 ODS column, eluted at 1 ml/min with gradient II: 20–50% D against C in 60 min, or gradient III: 50–80% D against C in 60 min (solvent C = 5% acetic acid; solvent D = 5% acetic acid/95% acetonitrile). T.l.c. used Whatman LK6D plates, developed with chloroform/methanol/acetic acid/water (30:7:1:1, by vol.). The relative abundance of each compound is given as the radioactivity incorporated into it relative to DIF-1.

Compound	H.p.l.c.				T.l.c. <i>R_F</i>	Relative abundance
	Step 1		Step 2			
	Elution time (min)	Gradient	Elution time (min)	Gradient		
DM8	36	I	21	II	0.54	1.6
DM7	40	I	23	II	0.58	1.0
DM6	40	I	26	II	0.60	0.7
DM4	40	I	29	II	0.68	3.3
DM5	46	I	39	II	0.39	0.7
DM2	62	I	8	III	0.69	0.5
DM3	62	I	10	III	0.80	0.3
CCD1	76	I	22	III	0.62	0.3
CCD2	80	I	30	III	0.62	0.5
CCD3	83	I	18	III	0.60	1.8
DIF-1	95	I	33	III	0.83	1.0
CCD4	109	I	44	III	0.57	3.1
CCD5	112	I	51	III	0.57	1.1
CCD6	115	I	56	III	0.58	3.5

Na^{36}Cl as indicated. At the appropriate times, labelled compounds were extracted with chloroform/methanol (Bligh & Dyer, 1959). Stalk and spore cells were separated by repeated sieving through a $100\text{ }\mu\text{m}$ mesh.

DIF activity was measured in a bioassay in which DIF induces isolated amoebae to differentiate into morphological stalk cells (Brookman *et al.*, 1982). Protein was measured by the Bradford dye-binding assay (Bio-Rad) against a BSA standard.

H.p.l.c. and t.l.c.

Where necessary, samples were concentrated by rotary evaporation or in a Speedvac concentrator. Small volumes of XAD-2 eluate could be loaded directly on to the h.p.l.c. apparatus, but larger extracts (from more than 10^{10} cells) were first 'cleaned up' by dilution to less than 10% ethanol, followed by binding to a C_{18} Sep-Pak (Waters) column and elution with 100% ethanol. H.p.l.c. was performed on a Varian 5000 or a Beckman System Gold machine, using Whatman or Spherisorb ODS packings as indicated. All gradients had a linear increase in the organic solvent and were either of methanol/water or acetonitrile/water, all in 2% or 5% acetic acid, as indicated in the text. ^{36}Cl radioactivity was counted and distinguished from ^3H in double-label experiments by standard scintillation-counting techniques.

T.l.c. was performed on Whatman LK6D silica plates, which were activated for 1 h at 100°C , loaded under N_2 and developed with chloroform/methanol/acetic acid/water (30:7:1:1, by vol.). Radioactivity was detected by autoradiography at -70°C on pre-flashed film, after the t.l.c. plates had been sprayed with En^3Hance (Amersham). The intensity of labelling of individual bands on the film was measured using a Molecular Dynamics scanning densitometer and compared with radioactive standards included on the plates.

Chemicals

Na^{36}Cl (6.7–19.3 μCi of Cl) was from Amersham. DIF-1 was synthesized as described by Masento *et al.* (1988), and [^3H]methoxy-DIF-1 (79 Ci/mmol) was a custom synthesis by Amersham. ^3H -labelled DIF-1 metabolites (DMs 2–8) were prepared and purified as described by Traynor & Kay (1991). Other reagents were of AR or h.p.l.c. grade as appropriate.

RESULTS

Labelling of developing cells with Na^{36}Cl and analysis of the labelled products

Cells, developing on a cellophane (i.e. dialysis) membrane, were incubated over a reservoir containing Na^{36}Cl in an otherwise chloride-free medium. Previous work has shown that DIF is efficiently absorbed from the medium of such cells by the non-polar resin XAD-2 (Kay *et al.*, 1983). XAD-2 resin was therefore included in the medium, separated from the cells by the dialysis membrane. In this way dialysable non-polar compounds released by the cells could be accumulated for analysis. Subsequent control experiments showed that the recovery of labelled compounds was very high by this method and that there did not appear to be any non-polar labelled compounds that were not absorbed by the resin. Labelling was performed with saturating Na^{36}Cl , which was found to be 0.05 mM or less.

Ax2 cells were allowed to develop in the continuous presence of Na^{36}Cl (1.8×10^{11} cells labelled with approx. 0.025 μCi of $\text{Na}^{36}\text{Cl}/\text{ml}$) until they had formed fruiting bodies. Under the conditions used for labelling this took about 2 days. The material

eluted from the XAD-2 resin was analysed by reverse-phase h.p.l.c. Elution with a methanol/water gradient resolved at least nine major labelled compounds (Fig. 1), some of which had associated stalk-cell-differentiation-inducing activity and some of which did not. Compounds have been named retrospectively as DIFs or DIF metabolites (DMs). DIF metabolites are numbered in the order in which they are believed to be made from DIF-1 (Traynor & Kay, 1991). Those labelled compounds yet to be identified are called 'chlorinated compounds from *Dictyostelium*' (CCDs). Each of the major peaks of radioactivity resolved in the first h.p.l.c. step was re-run on an acetonitrile/water solvent system giving a different selectivity. In some cases the peak from the first h.p.l.c. step was resolved into a number of separate labelled compounds by this second h.p.l.c. step (e.g. the groups of compounds DMs 4, 6 and 7, DMs 2 and 3 and CCDs 4, 5 and 6 were only clearly resolved from each other by the second h.p.l.c. step). In this way a total of 14 labelled compounds could be resolved; their chromatographic properties on both h.p.l.c. and t.l.c. are summarized in Table 1. For the number of ^{36}Cl -labelled compounds released, 14 is certainly a minimum estimate, as further very small peaks of radioactivity could also be detected, although these were not investigated further. Some of the compounds listed in Table 1 are considerably more abundant than DIF-1 itself on a c.p.m. basis; for instance, DM4 is about 2.5 times more abundant and CCD6 about 2.7 times more abundant than DIF-1.

Fig. 2. H.p.l.c. analysis of ^{36}Cl -labelled compounds produced at different times of development

Ax2 cells were set up for development on cellophane at $6 \times 10^5/\text{cm}^2$ and labelled with Na^{36}Cl ($0.02\text{ }\mu\text{Ci} \cdot \text{ml}^{-1}$) in DIFlab. The dialysable labelled compounds, released during the indicated periods of development and bound to XAD-2 resin, were analysed by h.p.l.c. under the same conditions as those used in Fig. 1. By 11 h the developing amoebae had formed mounds without tips, at 16 h first fingers and slugs predominated, at 21 h early culminates predominated and at 48–72 h mature fruiting bodies had formed.

Fig. 3. Time course of accumulation of ^{36}Cl -labelled compounds during *D. discoideum* development

(a) CCDs 4-6, which accumulate late in development (at the top of this panel are sketches of the organism's morphology at various times); (b) DIF-1 and its metabolites, which accumulate from aggregation onwards, and CCDs 1-3, one at least of which appears to accumulate during culmination. The h.p.l.c. conditions are as given in Fig. 1, and some of the h.p.l.c. separations are illustrated in Fig. 2. Compounds not well resolved on this h.p.l.c. system are combined here to give a single line. (a) $\diamond$, CCD4; $\square$, CCD6; (b) $\square$, DM4, 6, 7, 8; $\blacksquare$, DIF-1; $\blacklozenge$, DM5; $\blacksquare$, DM2, 3; $\blacklozenge$, CCD1, 2, 3.

Time course of chlorinated compound production during development

As a first step toward classifying the chlorine-labelled compounds, we determined when during development they are made. About 4×10^{10} cells were allowed to develop on cellophane over medium containing XAD-2 resin as described above, and at regular intervals the resin was changed. Labelled compound were eluted from the resin and analysed by h.p.l.c. It can be seen immediately (Figs. 2 and 3) that the released compounds fall into two or possibly three broad groups. A major group of compounds, subsequently shown to comprise DIF-1 and its metabolites, first accumulates at the end of aggregation and is then present until mature fruiting bodies have formed. A second group of three compounds (CCDs 4, 5 and 6), eluted after DIF-1 in the h.p.l.c. gradient, is only made very late in development, apparently by mature fruiting bodies. Finally, there appears to be a third group of compounds (CCDs 1-3) made with distinct kinetics: after the DIF-1 group, but probably before CCDs 4-6. Fruiting bodies also contain some very abundant cell-associated CCDs (see Fig. 4), some of which may be CCDs 4, 5 and 6, since they co-chromatograph on t.l.c. In addition there is at least one labelled compound in fruiting bodies which was not detected among the released compounds (CCD7 in Fig. 4). The cell-type specificity of the cell-associated compounds was investigated by extracting separated stalk and spore cells with chloroform/methanol. It is apparent from Fig. 4 that this late group of

Fig. 4. ^{36}Cl -labelled compounds are preferentially associated with the stalk rather than with the spores of the mature fruiting body

A band corresponding to DIF-1 is not visible in the t.l.c. of these extracts; the major labelled component has the same t.l.c. mobility as CCDs 4-6, though this identity has not been proved. There is also a component at R_f 0.87 that has no counterpart among the released compounds (Table 1) and is therefore called CCD7. Stalk and spores from mature fruiting bodies, which had developed on ^{36}Cl -containing agarose [Na^{36}Cl ($0.1 \mu\text{Ci} \cdot \text{ml}^{-1}$) in 10% DIFlab], were separated by filtration through a $100 \mu\text{m}$ mesh. Labelled compounds extracted with chloroform/methanol were separated by straight-phase t.l.c. and detected by autoradiography. The extract from 0.2 and 1 mg dry weight of each sample was run, together with a sample of the concentrated washings produced when the stalk and spores were separated. The autoradiograph was exposed for 4 days.

compounds is greatly enriched in the stalk cells; on a dry-weight basis they are at least tenfold more abundant in stalk than in spore cells.

Identification of some of the released compounds as DIF-1 and DMs

The peak of ^{36}Cl label at fraction 89 in Fig. 1 runs in the same place as a DIF-1 standard and is eluted with the major peak of stalk-cell-differentiation-inducing activity detected using the bio-assay. As already mentioned, it is also made at the same time of development as DIF-1. To confirm that the ^{36}Cl labelled peak is DIF-1, it was purified by another h.p.l.c. step and then shown to be co-eluted with authentic DIF-1 in two different h.p.l.c. solvent systems (Fig. 5) and a t.l.c. system (results not shown). It should be noted that, though the ^{36}Cl is co-eluted exactly with unlabelled DIF-1 (detected by u.v.), it is slightly retarded with respect to [^3H]methoxy-labelled DIF-1. This is presumably due to an isotope effect in which the substitution of ^3H for ^1H in the methoxy group slightly increases the polarity of the molecule. The same slight displacement is seen in all other comparisons of ^3H -labelled compounds with the corresponding ^{36}Cl -labelled or unlabelled compound. In a similar manner, DMs 2, 3, 4, 5 and 6 could be positively identified among the ^{36}Cl -labelled compounds, if allowance is made for the isotope effect. In the case of DM4, sufficient unlabelled material was also available for detection by u.v. As expected, the unlabelled material was co-

Fig. 5. Identification of ^{36}Cl -labelled DIF-1 (a) and DM-4 (b) by co-chromatography with the authentic compounds

The ^{36}Cl -labelled compounds were purified by two h.p.l.c. steps, the first of which is shown in Fig. 1, and then mixed with the appropriate authentic compound and chromatographed on a 25 cm Hichrom S5 ODS2 column at 1 ml/min using gradients of solvents A, B and E (A = 2% acetic acid; B = 2% acetic acid/98% methanol; E = 2% acetic acid/98% acetonitrile). (a) DIF-1, 60–85% B against A in 60 min; (b) DM-4, 10–45% E against A in 60 min. Fractions were collected every 0.5 min and the radioactivity and absorbance determined. It can be seen that the ^{36}Cl and unlabelled compounds are co-eluted exactly, but are slightly retarded with respect to the ^3H -labelled compounds. This result has been reproduced several times and is true for all other comparisons of ^3H and ^{36}Cl -labelled compounds. ■, ^3H ; □, ^{36}Cl ; ◆, A_{275} or A_{284} .

eluted exactly with the ^{36}Cl labelled peak suspected to be DM4 and slightly after the ^3H -labelled peak of DM4 (Fig. 5). DM7 and DM8 were identified with slightly less certainty, since there was only enough material for a single h.p.l.c. co-elution, though t.l.c. again supported the identities. All of these compounds are made co-ordinately during development with DIF-1 itself, as described above. A compound corresponding to DM1, the first metabolite produced from DIF-1, was also tentatively identified, though only a trace amount was present (result not shown). Thus it can be concluded that DIF-1 and all the major DMs previously identified (Traynor & Kay, 1991), are produced during normal development. However, a number of chlorinated compounds cannot be easily accounted for as DMs. First, there are three compounds which are eluted before DIF-1 on the h.p.l.c. gradient (CCDs 1–3), for which there is no corresponding metabolite. These compounds are probably made later in development than DIF-1 (see the above section). Secondly, there is a group of three compounds (CCDs 4–6), eluted after DIF-1, none of which can be a known DM. These compounds are made only very late in development, by mature fruiting bodies (see above) and presumably can have no role in development.

Chlorinated compounds in other slime-mould species

The methods of labelling chlorinated compounds described here are straightforward and quite sensitive. We used them to search for chlorinated compounds in other slime-mould species. Our choice of species was somewhat arbitrary, being determined by which would grow and develop adequately under the necessary laboratory conditions. *D. discoideum* (*D.d.*), is the standard

Fig. 6. Detection of a ^{36}Cl -compound metabolism in other species of slime moulds by t.l.c. autoradiography

A band with the mobility of DIF-1 is clearly visible in the mound/slug extracts of *D.d.*, *D.m.* and probably *P.v.*, and in the released compounds from *D.d.* and *D.m.* The released compounds were obtained from cells continuously labelled throughout development with Na^{36}Cl ($0.02 \mu\text{Ci} \cdot \text{ml}^{-1}$) in 10% DIFlab and collected after diffusion across a dialysis membrane and binding to XAD-2 non-polar resin. The other compounds were obtained by chloroform/methanol extraction of cells developing on agarose [containing Na^{36}Cl ($0.1 \mu\text{Ci} \cdot \text{ml}^{-1}$) in 10% DIFlab] to either the mound/slug stage (9.5–15 h of development, depending on strain) or until fruiting bodies were present (24–48 h of development). The material released by 10^8 cells ('Released' lanes) or extracted from 2 mg of cell protein ('Mound/slug' and 'Fruit' lanes, except for *P.v.*, where 0.5 mg was used) was loaded in each lane of the t.l.c. The autoradiograph was exposed for 190 days.

laboratory species included for comparison, *D. mucoroides* (*D.m.*) resembles *D.d.*, but the 'slug' lays down a stalk as it migrates; *D. vinaceo-fuscum* (*D.v.-f.*) also resembles *D.d.*, but is smaller and has a 'crampon-like' base; finally, *P. violaceum* (*P.v.*) represents another genus of cellular slime moulds and produces a fruiting body with small axial whorls of spores radiating from the main stalk. All species developed well in the conditions necessary for labelling, except for *D.v.-f.*, where only a minority of the amoebae developed all the way through to fruiting bodies. Three samples were analysed from each species: a chloroform/methanol extract of tipped mounds/slugs, a similar extract of mature fruiting bodies and the material released throughout development that could be collected across a dialysis membrane and bound to XAD-2 resin. It can be seen from Fig. 6 that all species tested produced chlorinated compounds during development and that a number of prominent compounds appear to be in common. A labelled band with the R_f of DIF-1 is clearly visible in the tipped-mound/slug extracts of *D.d.*, *D.m.* and *P.v.* and also in the XAD-2 extracts of *D.d.* and *D.m.* The XAD-2 material from *D.m.* was further analysed by h.p.l.c., and a labelled peak which co-eluted exactly with a DIF-1 standard was detected (Fig. 7). A similar experiment with the XAD-2 extract from *P.v.* also revealed a small peak with the mobility of DIF, but there was insufficient material for definitive co-elution experiments. These experiments

Fig. 7. Identification of DIF-1 in *Dictyostelium mucoroides*

(b) Total XAD-2 eluate (same material as that used in Fig. 1); (a) co-elution with authentic DIF-1 of the peak running in the position of DIF-1 in (b). H.p.l.c. was performed with a Hichrom S5 ODS-2 column eluted at 1 ml/min with 40–70% E against A in 60 min (A = 2% acetic acid; E = 2% acetic acid/98% acetonitrile). $\square$, ^{36}Cl ; $\blacklozenge$, A_{276} .

confirm the earlier detection of DIF in *D.m.* and *P.v.* using the bioassay (Brookman *et al.*, 1982) and prove beyond reasonable doubt that *D.m.* at least makes DIF-1. Although we did not detect DIF-1 in *D.v.f.* we cannot exclude its presence because of the poor development of this species under the labelling conditions. In addition, the mature fruiting bodies of *P.v.*, like *D.d.*, contain large amounts of at least two chlorinated compounds which are not present earlier in development. We therefore conclude that a developmentally regulated chlorinated-compound metabolism is probably widespread amongst slime-mould species of both Dictyostelid and Polysphondylid genera.

DISCUSSION

The objective of this work was to find a specific way of labelling DIF-1 and related compounds *in vivo* using ^{36}Cl . In this objective it has succeeded. During development, *D. discoideum* evidently releases a number of low-molecular-mass compounds which can be labelled with ^{36}Cl . Significant label is not incorporated into DIF-1 until the end of aggregation, and thereafter labelling increases rapidly to reach a peak at about the migrating-slug stage, before declining as the mature-fruiting-bodies form. This demonstrates that chlorine is incorporated into DIF-1 *de novo* from Cl^- during development and confirms the earlier developmental time courses for DIF accumulation, which relied on the stalk-cell-differentiation bioassay to measure DIF levels (Brookman *et al.*, 1982; Sobolewski *et al.*, 1983). All of the major labelled compounds accumulating at the same time as DIF-1 can be accounted for as DMs by the criterion of co-chromatography with authentic metabolites (where necessary making allowance for an isotope effect on the h.p.l.c. elution times). DIF-1 metabolism has so far only been studied by monitoring the fate of ^3H -labelled DIF-1 added to suspensions of developing cells (Traynor & Kay, 1991). Under these conditions, normal de-

velopment is disrupted, and it was therefore not certain that the pathway of metabolism with suspension cells would reflect that in normal development. DIF-1 metabolism by cells in suspension proceeds by a pathway in which the first three metabolites, DMs 1–3, are transitory, DMs 4 and 5 are more stable, and the more distal metabolites appear to be stable. DM1 is the only early metabolite in this pathway which has not definitely been identified by ^{36}Cl labelling. This is not surprising, since DM1 is normally cell-associated and only accumulates in the medium when high concentrations of DIF-1 are added to the cells. As expected, the more stable metabolites, DMs 4–8, were the major ones detected by ^{36}Cl labelling. This part of the work therefore provides evidence that the major steps in DIF-1 metabolism are the same with cells developing normally on a surface as with suspensions of cells metabolizing exogenous DIF-1.

A number of labelled compounds cannot be accounted for as DIF-1 or its metabolites. CCDs 1–3 were not resolved from each other in the time-course experiments, but it appears that at least one of them is made during culmination, somewhat later than the early group of compounds. Mature fruiting bodies produce a number of abundant chlorinated compounds. CCDs 4–6 are released into the medium, and compounds with similar R_F values are also abundant in stalk-cell extracts along with at least one additional compound (CCD7). Stalk cells have at least tenfold more of these compounds than spore cells on a dry-weight basis. Assuming that label is incorporated into the stalk compounds at the same specific radioactivity as it is added to the medium, a rough calculation suggests that covalent chlorine makes up about 1 part in 2500 of the dry weight of the stalk. Assuming one chlorine atom per molecule, this translates into at least 0.15 mM combined chlorinated compounds in the mature stalk. The role of these late compounds is unknown, but their abundance is reminiscent of plant secondary metabolites, and suggests that they may have some ecological role, such as aiding in spore dispersal or protection.

Labelling of natural products *in vivo* with ^{36}Cl has not been described before in any organism to our knowledge and may be useful in several ways. First, in *Dictyostelium*, it can be used to characterise mutants which may be defective in DIF-1 production or breakdown. We have already found that the strain HM44, which accumulates very little DIF-1 during development, does not produce appreciable amounts of any of the other chlorinated compounds (R. R. Kay, unpublished work). Secondly, it is possible to investigate cell-associated chlorinated compounds by t.l.c. and autoradiography, after labelling with ^{36}Cl and extraction with organic solvents (see Fig. 6). Unfortunately, owing to the low specific radioactivity of ^{36}Cl and the limited loading capacity of the t.l.c. plates, exposure times of at least 2 months are required to obtain a reasonable signal. Even so, one can determine which labelled compounds are present in the aggregate, their approximate concentrations and their spatial distributions (R. R. Kay, unpublished work). More generally this labelling procedure can be used to search for chlorinated-compounds metabolism in any organism or cell line for which a suitable labelling medium can be devised. Our experiments already show that chlorinated compounds are widespread, if not ubiquitous, among the cellular slime moulds and that at least one other species, in addition to *D. discoideum*, produces DIF-1.

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