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A MEMBRANE COMPONENT OF THE CELLULAR SLIME MOULD *DICTYOSTELIUM DISCOIDEUM* RAPIDLY LABELLED WITH [^{32}P]ORTHOPHOSPHATE

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SUMMARY

After labelling *Dictyostelium discoideum* (Strain Ax-2) for 30 min with [^{32}P]-orthophosphate a material was observed in cytoplasmic extracts, which cosedimented with polyribosomes in sucrose density gradients. The radioactivity in this material was insensitive to ribonuclease and deoxyribonuclease but was partially solubilized by alkali. All the radioactivity was rendered soluble in trichloroacetic acid by treatment with sodium deoxycholate. Most of the ^{32}P counts were extractable in chloroform–methanol (2:1, v/v) and upon analysis by thin-layer chromatography the isotope was found in various phospholipids, chiefly phosphatidylethanolamine with some in lecithin and phosphatidylserine. Upon examination in an electron microscope the material was found to be composed of membrane, glycogen and an unidentified amorphous material.

INTRODUCTION

The cellular slime moulds, *Dictyostelium discoideum* in particular, have been widely used in recent years for the detailed study of development and differentiation. They are ideally suited for this purpose as the growth phase of their life cycle is dissociated from the differentiation phase, enabling the two processes to be studied separately.

The importance of RNA metabolism for the control of differentiation has been widely recognized and several groups have investigated this using the cellular slime moulds. Radioactive labelling techniques have been employed generally using either uridine or uracil as an RNA precursor[1–6], though [^{32}P]orthophosphate has been used[7]. Phosphorus-containing compounds other than nucleic acids have not been extensively studied. The metabolism of nucleotides has been investigated[8]. The occurrence of phospholipids has also been studied as part of a larger investigation of slime mould lipids[9].

Abbreviation: BBOT, 2,5-bis(5′-*tert*-butylbenzoxazolyl-2′)thiophene.

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During the course of studies using [^{32}P]orthophosphate and *D. discoidieum*, a ^{32}P-pulse-labelled material sedimenting with polysomes on sucrose density gradients was observed. This material was unaffected by ribonuclease and for convenience will be referred to subsequently as Substance X. This paper describes Substance X in some detail and discusses its possible origins.

METHODS

Organism and growth conditions

The cellular slime mould *Dictyostelium discoideum* strain Ax-2 (ref. 10) was used throughout this work. It was cultivated in a medium resembling HL-5 Axenic medium[4] containing 14 mg/ml proteose peptone (Difco), 7 mg/ml bacto yeast extract (Difco) and 16 mg/ml glucose. The phosphate buffer was left out of the medium in order to improve the incorporation of [^{32}P]orthophosphate. The growth of the amoebae was unaffected.

Amoebae were grown axenically in this medium as follows: One or two spore heads from fruiting bodies were added by means of a sterile loop to 20 ml of medium in a 100-ml sterile erlenmeyer flask. The flask was incubated without shaking at 23 °C and after about 6 days contained $1 \cdot 10^6$–$2 \cdot 10^6$ organisms/ml (counted using a haemocytometer slide). Stock cultures were maintained by sub-culturing weekly. Stocks were not maintained by serial liquid passage for more than 6–8 weeks. At this time axenic cells were plated out on SM agar[11] in association with *Aerobacter aerogenes* and fresh stocks started from the spores produced.

Cells required for experimental purposes were incubated at 23 °C in an orbital incubator at 150 rev./min. Under these conditions the cells grew with a doubling time of 10–14 h to a final density of about $2 \cdot 10^7$ cells/ml. In the experiments to be described, exponentially growing cells were harvested at densities between $1 \cdot 10^6$ and $6 \cdot 10^6$ organisms/ml.

Preparation of lysates

Growing cells were poured on one quarter of their volume of frozen, crushed buffer containing 10 mM Tris, 50 mM KCl and 5 mM $MgCl_2$, adjusted to pH 7.5 with HCl at 22 °C. This was to stop any further incorporation of radioactive material present in the growth medium. The cells were then spun out and washed using the same buffer. The packed cell pellet was diluted 1:1 with buffer and made 0.3 % with respect to Triton X-100 (British Drug Houses) and then the suspension was stirred for 30 s on a Vortex mixer. The lysate was centrifuged at 10000 × *g* for 10 min to remove nuclei, debris, mitochondria *etc.*, and the supernatant was used for experiments (all operations were carried out at 5 °C). About 90 % cell breakage was achieved. The supernatant fraction (S10) obtained was largely cytoplasmic and free from nuclear contamination (as judged by microscopic examination and by the presence of the majority (90 %) of the cell DNA in the pellet fraction).

Sucrose density gradient analysis

Linear gradients were used ranging from 5–40 % (w/w) sucrose prepared in Tris–KCl–$MgCl_2$ buffer (pH 7.5). Preparative 29-ml gradients were centrifuged for 200 min using a Beckman SW 25.1 rotor at 24000 rev./min. Analytical 11.5-ml

gradients were centrifuged for 75 min using a Beckman SW 41 rotor at 41 000 rev./min. All centrifugations were carried out at about 2 °C in a Beckman Model L2-50HV ultracentrifuge.

After a gradient had been spun the bottom of the centrifuge tube was pierced with a needle and the contents of the tube pumped out, using a Palmer injection pump, through a recording spectrophotometer set to read at a wavelength of 260 nm (both Carey 15 and Hilger-Gilford spectrophotometers were used). Fractions from the gradient were collected using a fraction collector set for equal time intervals, or by counting drops.

Estimation of radioactivity

Most samples for radioactive counting were precipitated with 10 % (w/v) trichloroacetic acid. The precipitates were collected on Whatman GF/C glass fibre filters, washed twice with 5 % (w/v) trichloroacetic acid and three times with 1 % (v/v) acetic acid. They were then dried at 105 °C for 90 min and counted in a Packard Tri-Carb 3375 Liquid Scintillation Spectrometer using a toluene-based scintillation fluid containing 4 g/l of BBOT (2,5-bis(5′-*tert*-butylbenzoxazolyl-2′)thiophene) (C.I.B.A.). Liquid samples were suspended in a Triton X-100–toluene (1:2, v/v)-based scintillation fluid containing 10 % water and 4 g/l of BBOT. They were also counted in a liquid scintillation counter.

^{32}P-containing samples were also counted without scintillation fluid using Cerenkov radiation[12,13]. This had the advantage that gradients could be monitored for radioactivity without destroying the samples which could then be recovered for further analysis.

Thin-layer chromatography

Samples to be analyzed by thin-layer chromatography were resuspended in the Tris–KCl–$MgCl_2$ buffer (pH 7.5). This suspension was extracted twice with 5 vol. of chloroform–methanol (2:1, v/v) for 2 h at room temperature. The chloroform–methanol extract was then rotary evaporated and redissolved in about 2 ml of chloroform–methanol (19:1, v/v) saturated with water. This was layered on a 12 cm × 1 cm Sephadex G-25 column and was eluted with four column volumes of chloroform–methanol–water (19:1 v/v, satd). The column effluent was rotary evaporated and dissolved in chloroform ready for application to thin-layer plates.

All thin-layer chromatography was carried out on 18 cm × 18 cm glass plates coated with a layer of silica gel G (E. Merck A.G.), 0.25 mm thick. Before use the plates were activated by heating to 105 °C for 30 min. Samples (containing 4 μg of lipid phosphorus) were applied in 1-cm broad bands and chromatography was carried out, usually in two dimensions but occasionally in one dimension. The solvent systems used were those described by Rouser *et al.*[14]. Solvent I, chloroform–methanol–water (65:25:4, by vol.); Solvent II, chloroform–acetone–methanol–acetic acid–water (5:2:1:1:0.5, by vol.). Between solvents and on completion of chromatography the plates were dried at room temperature in air for 30 min.

Spots were detected using several sprays: (a) Molybdenum spray for the detection of phospholipids[15]. The blue colour appeared rapidly at room temperature. (b) Chromic acid charring spray for the detection of lipids and carbohydrates[16]. After the plate had been sprayed it was heated to 180 °C for 30 min, samples were charred

black. (c) Ninhydrin spray: a solution of 0.2 % ninhydrin in ethanol for the detection of lipids containing free amino groups. After the plate had been sprayed it was heated to 105 °C for 10 min to develop the colour.

Radioactivity on thin-layer plates was estimated in two ways. Autoradiograms were prepared by exposing Kodak BB54 Blue Brand X-ray film to the thin-layer plates. Alternatively the phospholipid-containing silica-gel samples were scraped into vials and the radioactivity counted in a scintillation counter using BBOT–toluene scintillant.

Methylated-albumin kieselguhr column chromatography

A methylated-albumin kieselguhr column was prepared as described by Mandell and Hershey[17]. The basal layer was made up from 8 g of kieselguhr (Hyflo supercel) supplemented with 2 ml of a 1 % (w/v) solution of methylalbumin; the middle layer contained 6 g of kieselguhr and 2 g of protein-coated kieselguhr; the upper layer was made up of 1 g of kieselguhr. The column was washed with 150 ml of 0.4 M NaCl in 50 mM phosphate buffer (pH 6.95) prior to use.

An S10 fraction of ^{32}P-labelled cells was prepared as usual. This fraction was made 2 % with respect to sodium dodecyl sulphate and was then extracted twice with equal volumes of phenol saturated with Tris–HCl (pH 7.5), followed by two extractions with chloroform–amyl alcohol (15:1, v/v). 2 vol. of ethanol were added to the aqueous phase which was left overnight at —20 °C. The precipitate which formed was dissolved in 0.4 M NaCl (in 50 mM phosphate buffer, pH 6.95) and was applied to the column. The column was then successively eluted at 23 °C as follows: (a) 150 ml 0.4 M NaCl, (b) 325 ml of a linear gradient from 0.4 to 1.0 M NaCl, (c) 200 ml 2.0 M NaCl (all saline solutions were made up in 50 mM phosphate buffer, pH 6.95).

The effluent from the column was led through an LKB Uvicord flow cell, which monitored the 254 nm absorbance, and 300-drop aliquots were collected, precipitated with trichloroacetic acid, filtered and counted for radioactivity as usual.

Electron microscopy

Samples for electron microscopy were resuspended in 50 mM Tris–HCl buffer (pH 7.5). The suspensions were negatively stained with 1 % (w/v) ammonium molybdate and were taken on to Formvar-coated copper electron microscope grids. The specimens were examined in an AEI Type EM801 electron microscope.

Analytical methods

The dry weight of cells was determined by drying cell suspensions to constant weight in an oven at 105 °C.

Protein was estimated by the method of Lowry *et al.*[18] using bovine serum albumin as standard.

Hexose was estimated by the anthrone method[19] using glucose as standard.

Lipid was estimated by extraction twice with chloroform–methanol (2:1, v/v) followed by evaporation to dryness *in vacuo* of the pooled extracts.

Lipid phosphorus was estimated by a modification of the method of Marinetti[20]. The sample was heated together with 0.9 ml of $HClO_4$ (specific gravity 1.70) for 20 min. The digestion flask was cooled, 7 ml of 1 M H_2SO_4 and 1.5 ml of 2.5 % (w/v) ammonium molybdate were added and mixed. 0.2 ml of Fiske-SubbaRow reagent

was added and the mixture was heated at 100 °C for 20 min, cooled and the absorbance at 820 nm read. Inorganic phosphate was used as standard.

Total nucleic acid was estimated by precipitating the sample with 0.5 M $HClO_4$ and then hydrolyzing twice (95 °C for 45 min). The $A_{260\ nm}$ of the resulting solution was read and nucleic acid estimated assuming that 100 μg of nucleic acid per ml had an $A_{260\ nm}$ of 2.8 (ref. 21).

Spectrophotometric measurements were carried out on Zeiss PMQ-II and Unicam SP500 instruments.

Fractionation of samples for radioactive analysis

Samples which had been labelled with [^{32}P]orthophosphate were fractionated into four parts as follows:

sample + 5 vol. chloroform–methanol (2 ×) ⟶ organic layer = lipid
↓
aqueous layer
+ 10% (w/v) trichloroacetic acid ⟶ supernatant = pool
+ bovine serum albumin
↓
precipitate
+ 10% (w/v) trichloroacetic acid ⟶ supernatant = nucleic acid
(95 °C for 45 min)
↓
precipitate = protein

Sometimes the chloroform–methanol extraction was omitted and only three fractions were obtained.

The lipid fractions were dried down and counted using BBOT–toluene scintillation fluid. The nucleic acid and pool fractions were extracted with ether to remove the trichloroacetic acid and were counted using BBOT–Triton X-100–toluene scintillation fluid. The protein samples were filtered and counted as usual.

Materials

Deoxyribonuclease I was obtained from the Worthington Biochemical Corporation. Ribonuclease A, alkaline phosphatase Type I and bacterial α-amylase Type II-A were obtained from the Sigma Chemical Company. Pronase (B grade) was obtained from Calbiochem Ltd. [5-^{3}H]Uridine (5 Ci/mmole) and [^{32}P]orthophosphate (high spec. act., neutralized with NaOH before use) were obtained from the Radiochemical Centre, Amersham.

RESULTS

Sucrose gradient analysis of ^{32}P-labelled supernatants

In bacterial systems ^{32}P-steady state-labelled cells have often been used in the analysis of polysomes[22]. The radioactivity profiles from sucrose density gradients of steady state-labelled cells show ribosomes and polysomes and resemble the absorbance profiles. The same was true of cellular slime moulds although ribonuclease never completely abolished the ^{32}P label in the polysomal region of the gradients, as it does in bacterial systems.

When cellular slime mould cells were labelled for short periods of time (40 min)

with [^{32}P]orthophosphate and S10 fractions prepared from them were analyzed on sucrose density gradients, a large amount of ^{32}P was found in the polysomal region (Fig. 1a). By analogy with bacterial systems this was assumed to be mRNA but on treatment with ribonuclease only 35 % of the radioactivity was solubilized (Fig. 1b). The ribonuclease-insensitive material was termed Substance X as described in the introduction.

The proportion of the pulse-labelled material in polysomes which was Substance X varied from experiment to experiment but it usually contained more than 50 % of the polysomal radioactivity and sometimes as much as 80 %. The remainder was presumably made up of mRNA and ribosomes. The latter only formed a small part of the labelled material as is shown by the similarity of the 80-S ribosomal radioactivity profiles before and after treatment with ribonuclease (Fig. 1b).

Time course of appearance of Substance X

The appearance of Substance X was followed using different pulse-times and spinning gradients in the presence of 25 μg/ml of ribonuclease (Fig. 2). Over the 3 h of the time course it was apparent that Substance X was labelled before the 80-S ribosomal peak but that after 3 h of labelling the 80-S region contained more radioactivity than Substance X. The spreading of the 80-S peak in the 1-h and 2-h samples was probably due to their standing for some time in the presence of ribonuclease which caused some breakdown of the ribosomes. Substance X, however, survived this treatment apparently unharmed, especially in the 2-h sample, suggesting that Substance X did not contain RNA or, if it did, that the RNA was well protected.

Effect of NaOH on Substance X

The effects of treating Substance X with alkali were investigated (Fig. 3). Ribonuclease removed 19 % of the ^{32}P radioactivity from the polysome region and

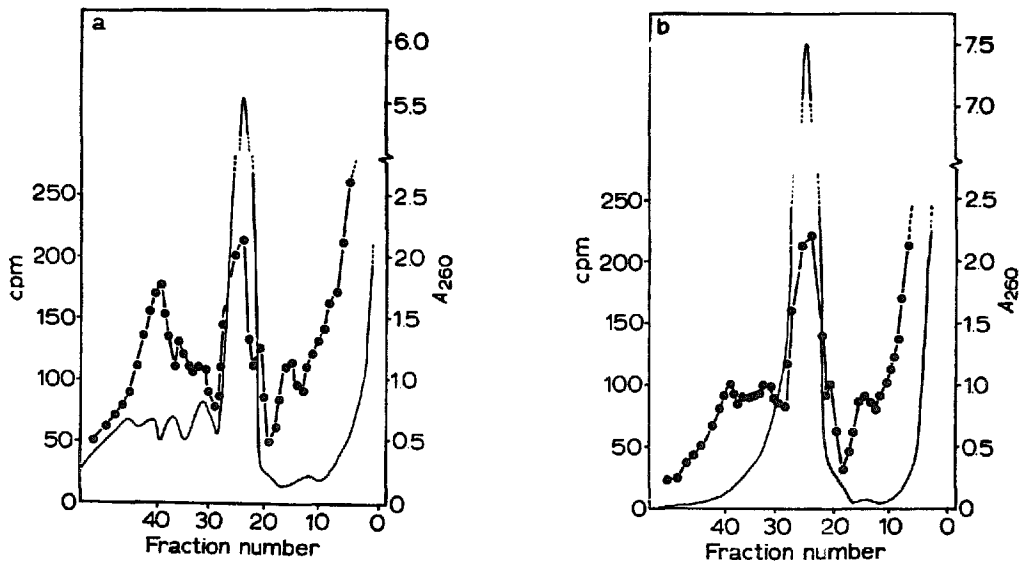

Fig. 1. Sucrose density gradient analysis of the S10 fraction from pulse-labelled cells. Cells growing exponentially at $2.2 \cdot 10^6$ organisms/ml were labelled with 2 μCi/ml [^{32}P]orthophosphate for 40 min. An S10 fraction was prepared and analyzed on sucrose density gradients. (a) No further treatment of S10. (b) S10 treated with 25 μg/ml ribonuclease before analysis. ●—●, radioactivity; ———, $A_{260\ nm}$.

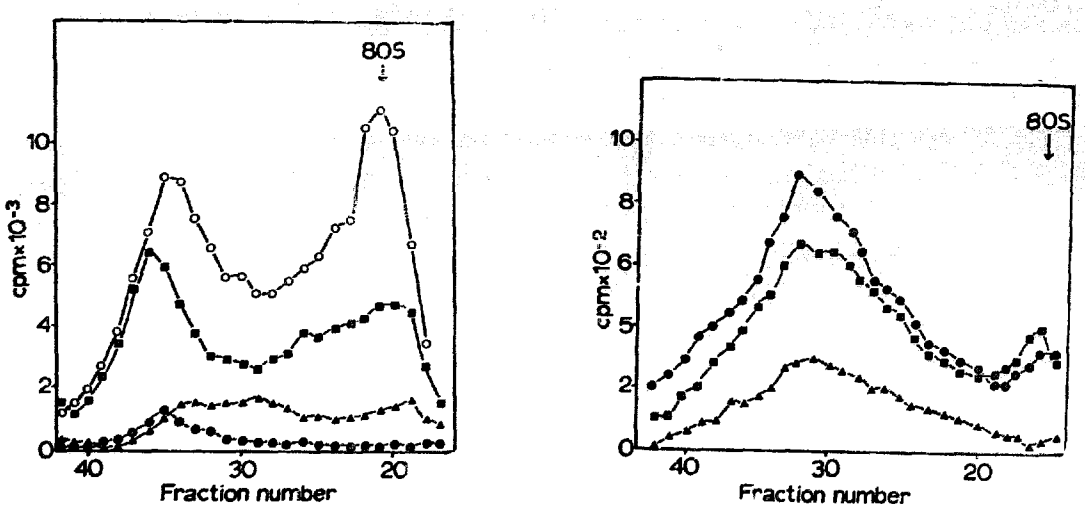

Fig. 2. Sucrose density gradient analysis of S10 fractions prepared from cells labelled for various times. Cells growing exponentially at $2.6 \cdot 10^6$ organisms/ml were labelled with 7 μCi/ml [^{32}P]-orthophosphate for 0.5, 1,2 or 3 h. S10 fractions were prepared and analyzed on sucrose density gradients after treatment with 25 μg/ml ribonuclease. The times of labelling were 0.5 h (●—●), 1 h (▲—▲), 2 h (■—■) and 3 h (○—○).

Fig. 3. Effect of NaOH upon the sucrose density gradient profiles of the S10 fraction from pulse labelled cells. Cells growing exponentially at $3.4 \cdot 10^6$ organisms/ml were labelled with 10 μCi/ml [^{32}P]orthophosphate for 30 min. An S10 fraction was prepared and analyzed on sucrose density gradients before (●) and after (■, ▲) treatment with 25 μg/ml ribonuclease. Two gradients (●, ■) were acid precipitated as usual. The samples from the third (▲) were treated with 1 M NaOH at 37 °C overnight before acid precipitation.

increased slightly the labelled material in the 80-S region. Thus, in this preparation, 81 % of the pulse label in the polysome region was in Substance X. Treatment of Substance X overnight (16 h) at 37 °C with 1 M NaOH solubilized 69 % of the ribonuclease-insensitive radioactivity. The labelled material in the 80-S peak was completely solubilized by this treatment, which was consistent with the radioactivity being in alkali-labile RNA. Thus, although Substance X was alkali-labile to some extent, it was not rendered soluble in trichloroacetic acid as readily as the rRNA of ribosomes.

Effect of an 'unlabelled chase' on Substance X

A batch of cells was labelled with [^{32}P]orthophosphate for 30 min and then the radioactivity was 'chased' for 60 min with 100 mM unlabelled phosphate, a concentration which was known to reduce considerably the uptake of ^{32}P. The sucrose gradient analysis of ribonuclease-treated lysates is shown in Fig. 4. It was immediately apparent that there was more labelled material after the chase than before it, and that the distribution of label had changed during the chase. There were several possible explanations for the 'extra' radioactivity: (a) some material which was soluble in trichloroacetic acid at 30 min might subsequently have been chased into precipitable material; (b) material which was present in cell nuclei at 30 min might have been chased into the cytoplasm and so have appeared in the S10 fraction; (c) the cells might have continued to incorporate [^{32}P]orthophosphate from the medium during the chase but at a reduced rate.

Possibilities (a) and/or (b) must have been occurring to some extent, for if

(c) had been the only mechanism operating the pulse and chase profiles should have had the same general shape, which they clearly did not.

At the end of the pulse-labelling period most of the radioactivity was in Substance X with a little in the ribosomal and subunit regions. On chasing with unlabelled phosphate the label in Substance X increased to some extent, especially in the region of lower sedimentation values, but the label in the ribosomal region increased much more dramatically (the total trichloroacetic acid-precipitable material on the gradient increased by 42 % whereas the increase in Substance X was only 25 %, but the increase in the ribosomal peak was 130 %). If it was assumed that little uptake of [^{32}P]orthophosphate occurred after the addition of unlabelled phosphate it was apparent that Substance X and ribosomes had different precursors.

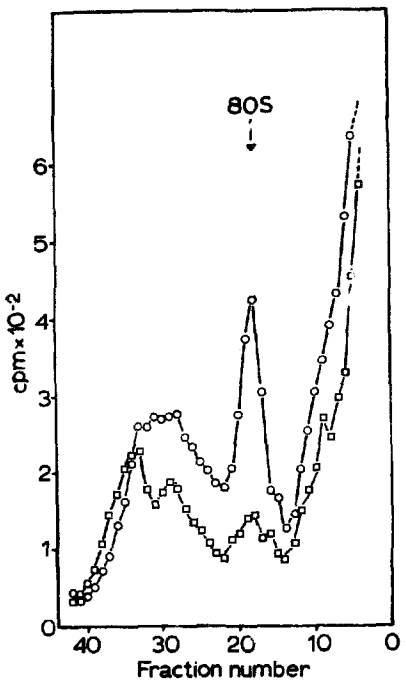

Fig. 4. Sucrose density gradient analysis of S10 fractions from cells pulse-labelled with [^{32}P]orthophosphate and subsequently treated with an 'unlabelled chase' of unlabelled orthophosphate. Cells growing exponentially at 3.10^6 organisms/ml were labelled with 8 μCi/ml [^{32}P]orthophosphate for 30 min (□—□) and were then treated with an 'unlabelled chase' of 100 mM phosphate (○—○). S10 fractions were prepared and analyzed on sucrose density gradients after treatment with 25 μg/ml ribonuclease.

Effect of various enzyme preparations on Substance X

A sample of Substance X was prepared using sucrose gradients followed by pelleting at high speed. The pellet was resuspended in Tris–KCl–$MgCl_2$ buffer (pH 7.5) and the effects of various enzymic treatments upon the trichloroacetic acid-precipitable ^{32}P radioactivity was determined (Table I). As expected ribonuclease had no effect; deoxyribonuclease had a small effect, reducing the radioactivity in Substance X by about 15 %. NaOH removed some label from Substance X but a considerable proportion of the radioactivity was stable to alkali. Pronase caused Substance X to lose about 40 % of its trichloroacetic acid-precipitable radioactivity. Pretreatment at 100 °C to denature proteins or addition of ribonuclease after Pronase treatment made no difference to these results. Treatment with alkaline phosphatase at pH 10

also reduced the trichloroacetic acid-precipitable radioactivity in Substance X by about 40 %. Steapsin, a lipase, reduced the radioactivity in Substance X by about 30 %.

TABLE I

EFFECT OF VARIOUS ENZYMES ON SUBSTANCE X

Samples of Substance X were prepared from ^{32}P-pulse-labelled lysates by pelleting sucrose density gradient fractions in a Beckman 50Ti rotor at 50000 rev./min for 150 min. The pellets were resuspended in Tris–KCl–$MgCl_2$ buffer (pH 7.5) and treated in various ways as detailed below. After treatment 200 μg of bovine serum albumin were added to each sample which was then acid-precipitated, filtered and counted.

Treatment	*cpm*	*% of control*
None	1300	100
0.7 M NaOH (20 h at 37 °C)	999	76
70 μg/ml RNAase (15 min at 23 °C)	1266	97
63 μg/ml DNAase (15 min at 23 °C)	1087	84
0.87 mg/ml Pronase (1 h at 23 °C)	784	60
0.37 mg/ml alkaline phosphatase (3 h at 37 °C; pH 10)	741	57
0.59 mg/ml steapsin (3 h at 37 °C)	875	67

None of the treatments set out in Table I removed all of the trichloroacetic acid-precipitable radioactivity from Substance X but Pronase, alkaline phosphatase and steapsin were all partially effective. This suggested that either the ^{32}P was in several different components which co-sedimented with polysomes by virtue of their size or that the ^{32}P was in only one component but that this was associated with other substances which rendered the radioactivity precipitable with trichloroacetic acid.

The effect of deoxycholate on Substance X

The effects of treating Substance X with deoxycholate, ribonuclease or a combination of the two are shown in Fig. 5. The effect of ribonuclease upon the polysomal radioactivity was the same as usual, about 35 % of the label being rendered soluble in trichloroacetic acid by the enzymic treatment. Deoxycholate had a much more dramatic effect removing 70 % of the total ^{32}P label in the polysomes. Together ribonuclease and deoxycholate removed 88 % of the ^{32}P from the pulse-labelled polysomes. Hence it appeared that a large proportion (and probably all) of Substance X was rendered trichloroacetic acid-soluble by treatment with the anionic detergent deoxycholate suggesting that Substance X consisted of membranous material or membrane enclosing the ^{32}P-labelled substance. The former was more likely as trichloroacetic acid precipitation on filters would almost certainly have washed out ^{32}P-labelled soluble material from inside membrane vesicles.

An experiment comparing the patterns of labelling produced by [^{32}P]orthophosphate and [5-^{3}H]uridine supported the hypothesis that the ^{32}P label in Substance X was not present in RNA (Fig. 6). The ^{32}P label in the polysome region behaved normally, ribonuclease removing 13 % of the label to leave Substance X. The ^{3}H label behaved completely differently, essentially all the polysomal label being removed by ribonuclease, consistent with the label being present in RNA. The addition of

deoxycholate to these gradients had little or no effect on the ^{3}H profiles and produced the same effects on the ^{32}P profiles as described above.

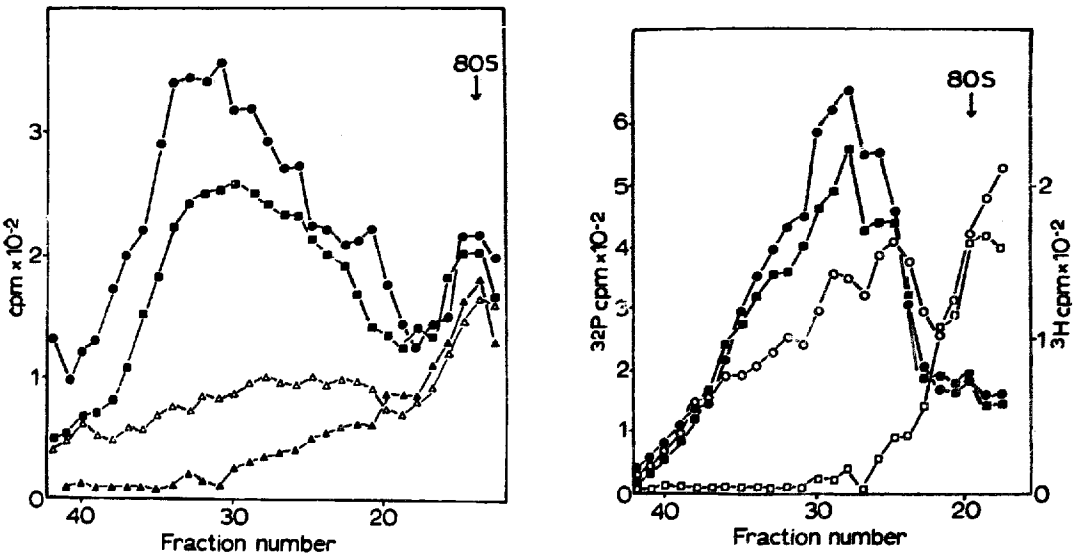

Fig. 5. Effect of sodium deoxycholate upon the sucrose density gradient profile of the S10 fraction from pulse-labelled cells. Cells growing exponentially at $3.3 \cdot 10^{6}$ organisms/ml were labelled with 12 μCi/ml [^{32}P]orthophosphate for 30 min. An S10 fraction was prepared and analyzed on sucrose density gradients after undergoing the following treatments; none (●), *plus* 0.5% sodium deoxycholate (△), *plus* 25 μg/ml ribonuclease (■), *plus* 25 μg/ml ribonuclease and then *plus* 0.5% sodium deoxycholate (▲).

Fig. 6. Sucrose density gradient analysis of the S10 fraction from cells doubly labelled with [^{32}P]-orthophosphate and [5-^{3}H]uridine. Cells growing exponentially at $6 \cdot 10^{6}$ organisms/ml were labelled for 30 min with 20 μCi/ml [5-^{3}H]uridine and 6 μCi/ml [^{32}P]orthophosphate. An S10 fraction was prepared and analyzed on sucrose density gradients before (●, ○) and after (■, □) treatment with 25 μg/ml ribonuclease. The radioactivity profiles are: ^{32}P: ■, ●; ^{3}H: □, ○.

Analysis of Substance X

Several batches of Substance X were prepared and analyzed for lipid, protein, nucleic acid and hexoses. There were substantial but variable amounts of lipid (33–56% of dry wt) and anthrone-positive hexose (25–82% of dry wt) present. There were also variable amounts of protein (5–34% of dry wt). There was, however, little or no nucleic acid in Substance X (1% of dry wt). The latter was confirmed by paper chromatography of alkaline and acid hydrolysates of Substance X. Only free [^{32}P]orthophosphate and no labelled nucleotides were detected. These results suggested that Substance X consisted of a mixture of several components which co-sedimented on sucrose density gradients but which varied in their relative proportions in different preparations.

Methylated-albumin kieselguhr column chromatography

The possible presence of polyphosphate in Substance X was studied using methylated-albumin kieselguhr columns. Polyphosphate can be extracted by the sodium dodecyl sulphate–phenol nucleic acid preparative method and when applied to methylated-albumin kieselguhr columns is eluted between the tRNA and rRNA peaks[23]. The analysis of a total nucleic acid extract from the S10 fraction of ^{32}P-pulse-labelled cells is shown in Fig. 7. The 0.4 M NaCl removed tRNA, degraded RNA and

unincorporated ^{32}P (large amounts of trichloroacetic acid-soluble radioactivity were present in these early fractions). The NaCl gradient eluted the bulk RNA which was visible as a large absorbance peak. All the radioactivity was found associated with the tRNA and bulk RNA; none was found between the two absorbance peaks, the elution position of polyphosphate.

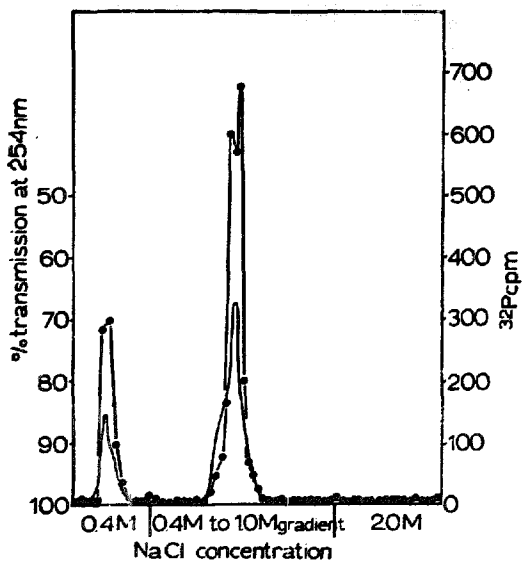

Fig. 7. Methylated-albumin kieselguhr column chromatography of nucleic acid fraction, pulse-labelled with [^{32}P]orthophosphate. Cells growing exponentially at $1.5 \cdot 10^6$ organisms/ml were labelled with 9 μCi/ml [^{32}P]orthophosphate. An S10 fraction was prepared and nucleic acid extracted from it as described in Methods. The extract was analyzed using a methylated-albumin kieselguhr column. The effluent from the column was monitored for 254 nm absorbance (———) and radioactivity (●—●).

These results clearly showed the absence of labelled polyphosphate from cytoplasmic extracts of pulse-labelled cellular slime moulds. As Substance X was a component of such extracts they suggested that Substance X was not composed of polyphosphate. No attempt was made to investigate the presence or absence of polyphosphate in steady state-labelled cells and so the results above do not rule out the possibility that cellular slime moulds, like slime moulds (*Physarum polycephalum*) contain polyphosphate. They do show, however, that any such polyphosphate, if it exists, cannot be found pulse-labelled in cytoplasmic extracts. Indeed polyphosphate in *P. polycephalum* is only found in nuclei and the 30000 × *g* pellet fractions and is absent from cytoplasmic supernatants[23].

Fractionation of Substance X

Preparations of Substance X were fractionated as described in the Methods section with and without lipid extractions. 'Ribonuclease-treated polysomal' fractions from steady state-labelled cells were also fractionated (Table II). Of the ^{32}P label present in Substance X 80–85 % was trichloroacetic acid-precipitable, acid hydrolysis rendering about 60 % of this soluble (nucleic acid fraction). However, if a lipid extraction was carried out prior to acid precipitation only 4–12 % of the label was subsequently precipitable, 65–68 % of the ^{32}P having been removed in the chloroform–methanol extract. Clearly, about 80 % of the trichloroacetic acid-precipitable material present in Substance X was extractable into chloroform–methanol. This

suggested that the ^{32}P in Substance X must have been associated very closely with lipid and in all probability was present in phospholipid. Similar results were obtained with extracts from steady state-labelled cells (Table II).

TABLE II

LOCALIZATION OF ^{32}P LABEL IN SUBSTANCE X

Samples of Substance X from pulse-labelled cells and from 'ribonuclease-treated polysomal fraction' of steady state-labelled cells were prepared from sucrose density gradients and were fractionated as described in Methods. The results from two different experiments are presented.

Fraction	*% of total cpm in fraction*					
	Pulse-labelled material (no chloroform–methanol extraction)		*Pulse-labelled material (chloroform–methanol extraction)*		*Steady state-labelled material (chloroform–methanol extraction)*	
Lipid	—	—	65	68	76	67
Pool	20	15	31	20	18	26
Nucleic acid	46	53	3	9	2	3
Protein	34	32	1	3	4	4

Electron microscopic examination of Substance X

The appearance of Substance X in the electron microscope after various treatments is shown in Fig. 8. Substance X was seen to be composed of several distinct structures (Figs 8a and 8b): M, large sheets of membrane-like material varying considerably in size; G, granular particles of a fairly regular size, 25–40 nm in diameter, which appeared to be made up of smaller subunits. This is clearly shown in Fig. 8a: A, irregular amorphous material of varying size, sometimes fibrillar in nature. It is possible that this material was derived from the membrane sheets by the breakdown of their edges as indicated by the arrows on Fig. 8b.

The presence of large amounts of membrane-like structures in these preparations was reassuring and provided a possible source for the ^{32}P-labelled, lipid-extractable material in Substance X. As expected, there were no ribosomes in these fractions.

When preparations were derived from deoxycholate-treated lysates colourless pellets were produced (reddish pellets were normally obtained) and on examination (Fig. 8c) they were found to contain only large amounts of the amorphous material (A) and the granular material (G). There were no membrane sheets at all.

The identity of the granular material was unknown but it had a similar appearance and size to α-glycogen as isolated from *Tetrahymena pyriformis*[24]. Glycogen is known to exist in varying degrees of complexity[25] and under the electron microscope has a characteristic subunit structure which is clearly visible in negatively stained samples. There was thus a distinct possibility that the granular structures were glycogen and as α-glycogen from *T. pyriformis* has a sedimentation coefficient of 214[24] (about the same as Substance X) this was investigated. A preparation of Substance X was derived from a lysate which had been treated with α-amylase. The preparation contained only membrane (M) and amorphous material (A) (Fig. 8d). It seemed likely, therefore, that Material G was glycogen. This is known to be the major carbohydrate in cellular slime mould cells grown axenically in the presence of glucose[26] and, in rat liver preparations, it co-sediments with polysomes[27]. If G was

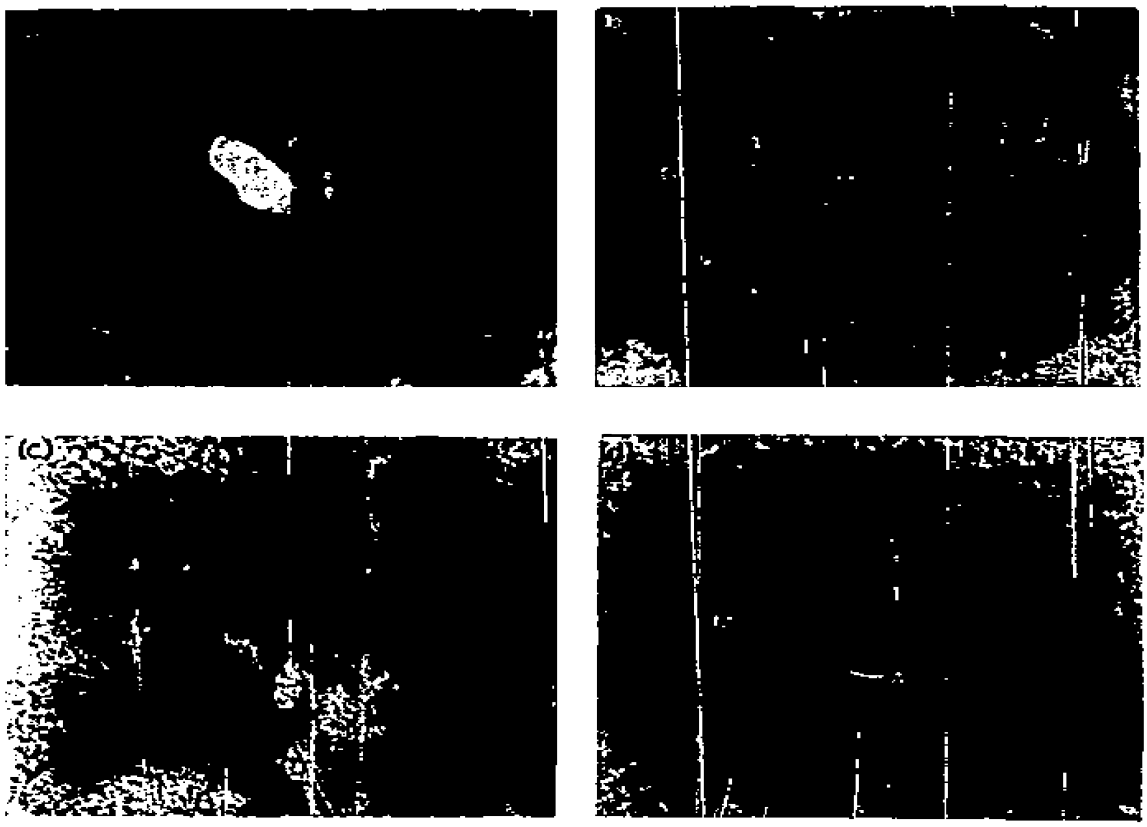

Fig. 8. Electron micrographs of negatively stained samples of Substance X after various treatments. The symbols represent: A, amorphous material; M, membrane-like structures; G, granular material. The bar represents 100 nm. (a) Untreated sample of Substance X. (b) Untreated sample of Substance X; arrows indicate possible origin of amorphous material from disintegrating membrane. (c) Substance X pretreated with 0.5% sodium deoxycholate. (d) Substance X pretreated with 200 units/ml of α-amylase (25 °C for 10 min).

glycogen this would account for the high levels of anthrone-positive material detected in Substance X. Variations in the relative proportions of glycogen, membrane and amorphous material in Substance X could also account for the variations in the composition of Substance X.

Thin-layer chromatography

The material extracted into chloroform–methanol (2:1, v/v) was further analyzed utilizing thin-layer chromatographic techniques. Four types of ^{32}P-labelled sample were compared: PXL, pulse-labelled Substance X lipid; PCL, pulse-labelled whole cell lipid; SXL, steady state-labelled Substance X lipid; SCL, steady state-labelled whole cell lipid.

The samples were analyzed by two-dimensional thin-layer chromatography (Fig. 9). PXL produced five major spots, two of them ninhydrin-positive. Phosphatidylcholine and phosphatidylserine were often overlapping and hence separation of the radioactivity was difficult. Phosphatidylethanolamine was the major dark spot on autoradiograms, containing about 40% of the total ^{32}P radioactivity. The next most radioactive area was that occupied by phosphatidylcholine and phos-

phatidylserine which accounted for about 30% of the radioactivity. PCL produced a similar pattern. SXL also produced a similar pattern but a sixth spot, A, possibly lysophosphatidylcholine, which had been seen in sample PXL as a faint streak, was clearly visible. Again the major radioactive phospholipid was phosphatidylethanolamine, containing about 50% of the ^{32}P. SCL produced a similar pattern to PCL.

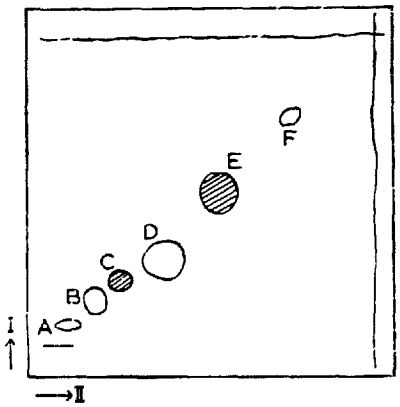

Fig. 9. Thin-layer chromatogram of lipid preparations. I and II refer to the solvent systems described in Methods. The plates were run in Solvent I before Solvent II. The spots depicted are those produced by the molybdenum–phosphorus spray. The cross-hatched spots were also stained with ninhydrin. The individual spots are: A, lysophosphatidyl choline?; B, ?; C, phosphatidylserine; D, phosphatidylcholine; E, phosphatidylethanolamine; F, cardiolipin?

TABLE III

^{32}P-LABELLED LIPID PREPARATIONS

Various ^{32}P-labelled lipid preparations (PXL, SXL, PCL, SCL; see text) were compared by thin-layer chromatography. The molybdenum–phosphorus stained spots were counted for radioactivity. The various phospholipids are those shown in Fig. 9. The results are the mean of three experiments together with the S.E.

Phospholipid	*% of total cpm in phospholipid*				*% of total phospholipid (ref. 9)*
	PXL	*SXL*	*PCL*	*SCL*	
Lysophosphatidylcholine?	6 ± 3	3 ± 1	8	2 ± 1	—
Phospholipid B	6 ± 2	4 ± 1	5 ± 2	1 ± 1	—
Phosphatidylserine + phosphatidylcholine	28 ± 2	25 ± 2	19 ± 3	36 ± 1	41
Phosphatidylethanolamine	40 ± 2	48 ± 3	45 ± 3	45 ± 2	34
Cardiolipin?	2 ± 1	5 ± 1	2 ± 1	3 ± 1	8
Lysophosphatidylethanolamine	—	—	—	—	10
Phosphatidalethanolamine	—	—	—	—	8

The various ^{32}P-labelled preparations were compared quantitatively by running the samples in one dimension in Solvent I, scraping off the various molybdenum–phosphorus stained spots and counting them in a liquid scintillation counter. The results from these experiments are set out in Table III. It was apparent from these results that in all samples phosphatidylethanolamine was the most radioactive spot containing between 40 and 50% of the total radioactivity. Although it appeared to be labelled more rapidly than phosphatidylcholine and phosphatidylserine in whole

cells this did not appear to be so in Substance X. In steady state-labelled cells, 45 % of the ^{32}P was in phosphatidylethanolamine and 36 % in phosphatidylcholine and phosphatidylserine. Thus, approximately 45 % of the phospholipid in whole cells was phosphatidylethanolamine and 36 % was phosphatidylcholine and phosphatidylserine. However, only 19 % of pulse-labelled ^{32}P was in phosphatidylcholine and phosphatidylserine whereas 45 % of the pulse-labelled ^{32}P was in phosphatidylethanolamine. Hence, the 'specific activity' of phosphatidylethanolamine in pulse-labelled cells was 1 (45/45) whereas that of phosphatidylcholine and phosphatidylserine was 0.53 (19/36). In Substance X the 'specific activities' were approximately the same after pulse labelling.

The fact that steady state-labelled cells had more radioactive phosphatidylethanolamine than phosphatidylcholine and phosphatidylserine (and hence had more phosphatidylethanolamine than phosphatidylcholine and phosphatidylserine) was surprising as Davidoff and Korn[9] had reported that the opposite was the case. There were several possible explanations for this; firstly, their extraction procedure was different from the procedure used here; secondly, they used a different strain of the organism grown in a different way and it was possible that nutritional conditions could have varied the lipids of the cells considerably; and, thirdly, they reported the presence of two other ethanolamine-containing phospholipids which they suggested could have been artefacts produced during isolation or storage.

DISCUSSION

The presence of a ribonuclease-insensitive, ^{32}P-labelled material in lysates from cellular slime moulds has been described. The material (Substance X) was found sedimenting in the polysome region of sucrose density gradients but could not have been RNA for various reasons. It was synthesized from different precursors by a different route as shown by the time-course and 'pulse-chase' experiments. Substance X was much less sensitive to alkaline degradation than ribosomes and was, as mentioned above, insensitive to ribonuclease. The sensitivity of the material to various other enzymes was quite unlike that of RNA. The radioactivity profiles obtained after labelling cells with [5-^{3}H]uridine, which was known to label RNA specifically, were considerably different from those obtained using [^{32}P]orthophosphate. No nucleic acid could be detected by direct analysis of samples of Substance X. The possibility that the ^{32}P label was present in polyphosphate was eliminated by the results obtained using methylated-albumin kieselguhr columns.

Another possible location for ^{32}P was in phospholipids contained in membrane material co-sedimenting with polysomes. This was confirmed in several ways. The radioactivity was extractable in chloroform–methanol and in the presence of the detergent sodium deoxycholate the radioactivity profiles on sucrose density gradients were essentially the same whether the cells were labelled with [^{32}P]orthophosphate or [5-^{3}H]uridine. Extraction in chloroform–methanol and analysis by thin-layer chromatography showed the presence of several radioactively labelled phospholipids. Examination in an electron microscope revealed, as well as the expected membrane fragments, considerable quantities of glycogen and an unidentified amorphous material. There were no ribosomes. On treatment with sodium deoxycholate the ^{32}P radioactivity was removed from samples of Substance X and the membrane fragments

also disappeared suggesting that the radioactivity was indeed present in the membrane phospholipids.

The presence of several components in the samples of Substance X explained the variable composition of Substance X and also the effects of the various enzyme treatments.

Substance X was not an artefact of the lysis method for both homogenization of the cells and the use of another non-ionic detergent Cemulsol NPT-12 produced similar results. The presence of radioactivity in particulate membrane material was further supported by centrifuging at 30000 $\times$ *g* for 30 min when most of Substance X was removed but normal polysome profiles were obtained.

Thus, the presence of a rapidly labelled membrane fraction, in cellular slime moulds has been described. It is possible that this may have been a specialized membrane fraction or it may merely have been a random collection of small fragments. Its association with the polysomes at first suggested that the membrane was pieces of rough endoplasmic reticulum. This was not likely, however, for both high speed centrifugation and sodium deoxycholate removed the membrane fraction without markedly affecting the polysomal profiles. However, the difference found in specific radioactivity of Substance X lipids and whole cell lipids suggested that the membrane pieces may not have been random fragments. This may have been a reflection of the fact that the membranes observed were a Triton X-100-resistant 'core'.

The experiments described also suggest that care must be taken with some microorganisms, as with rat liver *etc.*, if glycogen co-sedimenting with polysomes is not to cause problems. This trouble is acute in axenically grown cellular slime moulds cultivated on glucose, which contain large amounts of this polysaccharide[26].

This paper has been, in a way, a cautionary tale about the use of radioactive precursors and sucrose density gradients. It has illustrated how it would be possible to be misled by not checking the identity of labelled materials found in cells which have been grown in the presence of radioactive compounds. It has also shown how sucrose density gradient analysis can be very misleading if the material placed on the gradient is not what it is imagined to be. If the ^{32}P-labelled material found in the experiments described here had been assumed to be nucleic acid by analogy with bacterial systems[22] the conclusions drawn would have been completely wrong. Hence, the importance of checking which materials have been labelled by radioactive precursors cannot be overstressed, even if the system used resembles others which have already been investigated.

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REFERENCES

1 R. G. Pannbacker, *Biochem. Biophys. Res. Commun.*, 24 (1966) 340.
2 J. Inselburg and M. Sussman, *J. Gen. Microbiol.*, 46 (1967) 59.
3 C. Ceccarini, M. S. Campo and F. Andronico, *J. Mol. Biol.*, 54 (1970) 33.
4 S. M. Cocucci and M. Sussman, *J. Cell Biol.*, 45 (1970) 399.
5 Y. Mizukami and M. Iwabuchi, *Exp. Cell Res.*, 63 (1970) 317.
6 M. Iwabuchi, Y. Mizukami and M. Sameshima, *Biochim. Biophys. Acta*, 228 (1971) 693.
7 R. R. Sussman, *Biochim. Biophys. Acta*, 149 (1967) 407.
8 C. L. Rutherford and B. E. Wright, *J. Bacteriol.*, 108 (1971) 269.
9 F. Davidoff and E. D. Korn, *J. Biol. Chem.*, 238 (1963) 3199.
10 D. J. Watts and J. M. Ashworth, *Biochem. J.*, 119 (1970) 171.
11 M. Sussman, in D. M. Prescott, *Methods in Cell Physiology*, Vol. 2, Academic Press, London, 1966, p. 397.
12 T. Clausen, *Anal. Biochem.*, 22 (1968) 70.
13 R. T. Haviland and L. L. Bieber, *Anal. Biochem.*, 33 (1970) 323.
14 G. Rouser, G. Kritchevsky and A. Yamamoto, in G. V. Marinetti, *Lipid Chromatographic Analysis*, Vol. 1, Edward Arnold, London, 1967, p. 99.
15 J. C. Dittmer and R. L. Lester, *J. Lipid Res.*, 5 (1964) 126.
16 G. Rouser, C. Galli, E. Lieber, M. L. Blank and O. S. Privett, *J Am. Oil Chem. Soc.*, 41 (1964) 836.
17 J. D. Mandell and A. D. Hershey, *Anal. Biochem.*, 1 (1960) 66.
18 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
19 J. E. Hodge and B. T. Hofreiter, in R. L. Whistler and M. L. Wolfram, *Methods in Carbohydrate Chemistry*, Vol. 1, Academic Press, London, 1962, p. 380.
20 G. V. Marinetti, *J. Lipid Res.*, 3 (1962) 1.
21 E. F. Gale and J. P. Folkes, *Biochem. J.*, 53 (1953) 483.
22 E. Cundliffe and K. McQuillen, *J. Mol. Biol.*, 30 (1967) 137.
23 H. W. Sauer, K. L. Babcock and H. P. Rusch, *J. Bacteriol.*, 99 (1969) 650.
24 A. A. Barber, W. W. Harris and G. M. Padilla, *J. Cell Biol.*, 27 (1965) 281.
25 P. Drochmans, *J. Ultrastruct. Res.*, 6 (1962) 141.
26 J. M. Ashworth and D. J. Watts, *Biochem. J.*, 119 (1970) 175.
27 A. Korner, *Biochem. J.*, 81 (1961) 168.