The fraction with the second largest accumulation of carotene is the 4,900×*g* sediment. There was a similar relative distribution of carotene, total lipids, protein and dry solids when the mycelium was grown with 1% lipids in the medium. However, comparatively less lipid and more protein and dry solids sedimented in the 600×*g* fraction (Table). It seems that the mycelium grown at the lower lipid level is more resistant to disruption, which may be a reflection of differences in proportions of carbohydrates and lipids in the mycelium cell wall.

The distribution of carotene in relation to dry solids, protein and total lipids using mycelium grown with 4% or 1% lipids in the medium is shown in Figure 1. In the first mentioned case all 3 parameters have a distinct peak at 4,900×*g*. The pattern is quite different in fractions prepared from mycelium grown with 1% lipids in the medium. The peaks at 4,900×*g* are missing, while the carotene : lipid ratio in the 600×*g* sediment is very high (Figure 1B).

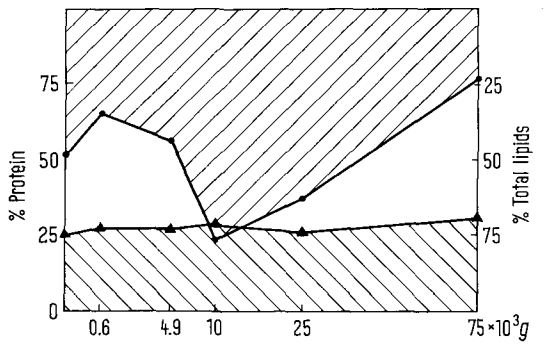

Fig. 2. Contents of protein (▲—▲) and total lipids (●—●) expressed as a percentage of dry solids, in subcellular fractions of *B. trispora*. Medium containing 4% lipids.

Figure 2 shows the contents of total lipids and protein in relation to dry solids in the various sediments. There is a minimum in the lipid content of the 600×*g* sediment which may be explained by the aforesaid ejection of 'free' lipids during centrifuging. The large content of lipids in the 10,000×*g* fraction probably corresponds to light mitochondria. The pattern was essentially similar using mycelium grown with 1% lipids, but the lipid content of all fractions was lower.

It appears from these data that there are at least 2 pools of β-carotene in the mycelium of *B. trispora*. One of them is associated with the fraction sedimenting at 4,900×*g*, presumably consisting mainly of cell walls and heavy mitochondria. There also seems to be a large pool of free β-carotene, dissolved in fat globules suspended in the cytoplasm. Similar fat inclusions are considered to be the main pool of carotenoids in fungi[4]. The magnitude of this pool in *B. trispora* may be related to the excessive synthesis of β-carotene which, as pointed out above, is substantially increased in the presence of exogenously supplied lipids[5].

Résumé. On a obtenu 2 pools majeurs de β-carotène dans l'homogénat du mycélium de *B. trispora*. L'un d'eux est associé à la fraction sédimentable à 4,900×*g*, l'autre aux globules de matière grasse, dans le cytoplasme.

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[5] Acknowledgements. This investigation was supported by The Swedish Board of Technical Development Grant No. TFR4325 and The Swedish Agricultural Research Council, Grant No. A1628/B1219 to HALINA Y. NEUJAHR.

Microbiological Assay of Cyclic 3′, 5′-AMP

Cyclic 3′, 5′-AMP is present in many tissues of various animals, where it may act as a 'second messenger'[1]. Several biochemical assays for cyclic 3′, 5′-AMP are available. A microbiological assay for qualitative and quantitative determination of cyclic 3′, 5′-AMP is described in this report using myxamoebae which move towards sources containing this compound. This method shows high specificity and sensitivity. An additional advantage is that all kinds of extracts may be assayed for cyclic 3′, 5′-AMP without previous purification procedures.

Materials and methods. The preparation of myxamoebae, a hydrophobic agar surface and the measurement of its rigidity and hydrophoby have been described previously[2,3]. Using a hydrophobic agar of a suitable rigidity the cells stayed inside the drops unless an active attractant outside the drops induces them to pass the boundary (Figure 1). 100–150 drops (each 0.1 µl) of a myxamoebae suspension were placed on such an agar. Every responding population (drop diameter ca. 0.6 mm) contained 500–1000 cells.

Myxamoebae were most sensitive to attractants at the onset of aggregation[4,5]. Small populations of myxamoebae, incubated at 22 °C in darkness, aggregated 8–10 h after deposition. The sensitivity of the cells in the various drops was better synchronized when they were incubated at 22 °C for 2–3 h, stored overnight at 5–6 °C, and used the next day. A sudden change in temperature may sometimes lead to withdrawal of pseudopods, especially at 25 °C or higher. Incubation at 16 °C for 30–60 min before exposure to room temperature prevented rounding-up of the cells. Drops (0.1 µl) of an active extract were placed near (100–500 µm away) the responding drops. The attraction was observed 30 or 45 min after deposition, through a phase contrast microscope (× 80). The cells in the responding populations should not be aggregating at the time of observation. A response was scored positive

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when myxamoebae moved across the margin of the responding drop (Figure 1).

Extracts that induced a very weak response were deposited 3–5 times at 5 min intervals, imitating the pulses that are characteristic of aggregating myxamoebae. Observation took place 5 min after the last deposition.

Results and discussion. Active sources are bacteria and aggregates of myxamoebae (Figure 1). The attractant, secreted by these organisms, was shown to be adenosine 3′,5′-monophosphate (cyclic 3′,5′-AMP)[6–10]. At high cyclic 3′,5′-AMP concentrations, as with a concentrated water extract of bacteria[4], the myxamoebae crossed the margins at all sides (Figure 2). The presence of extracellular phosphodiesterase in myxamoebae populations may explain why cells also moved away from the attracting source. Myxamoebae secrete phosphodiesterase[11], which inactivates cyclic 3′,5′-AMP[12,13]. Presumably this enzyme diffused through the agar and inactivated the cyclic 3′,5′-AMP around the drop, creating a steep gradient of cyclic 3′,5′-AMP in all directions away from the centre. This steep gradient activated the cells to move outside and away from the drop over distances as much as 0.8 mm from the edge. If phosphodiesterase is inducible by cyclic 3′,5′-AMP the increased production of the phosphodiesterase induced by the high concentration of cyclic 3′,5′-AMP in the agar, could explain why amoebae move so far away.

At lower concentrations of cyclic 3′,5′-AMP the responding myxamoebae detected the gradient of the attractant and only moved towards the attracting drop (Figure 1). At very low concentrations of cyclic 3′,5′-AMP in the extracts, myxamoebae did not cross the edge of the drop, but stayed inside, pressed against the margin. Such a response was considered positive when at least twice as many cells were pressed against the margin closest to the attracting drop as on the opposite side (Figure 3). Amounts as low as 10^{-12} g of cyclic 3′,5′-AMP elicited such a response.

The concentration of cyclic 3′,5′-AMP in an active extract was measured by dilution of the extract until the concentration becomes so low that not all but only a certain percentage of the responding populations reacted positively. The cyclic 3′,5′-AMP concentration in the extract was estimated by evoking a similar percentage of positively responding populations with known concentrations of commercial 3′,5′-AMP. Two-fold differences in cyclic 3′,5′-AMP concentrations were demonstrated in this way.

Many other compounds were tested; only 3′,5′-cyclic nucleotides and some of their analogues were active[8]. The 3′,5′-ring seems to be indispensable for attraction. The concentration of cyclic nucleotides, other than cyclic 3′,5′-AMP, needed to be much higher to elicit a response.

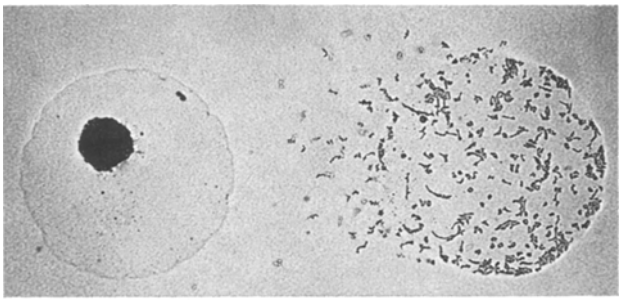

Fig. 1. Chemotaxis in *Dictyostelium discoideum*. The aggregation in the drop on the left attracts myxamoebae outside the boundary of the drop on the right. Cells inside the drop move on the agar surface. Cells outside the drop crawl through the agar. × 60.

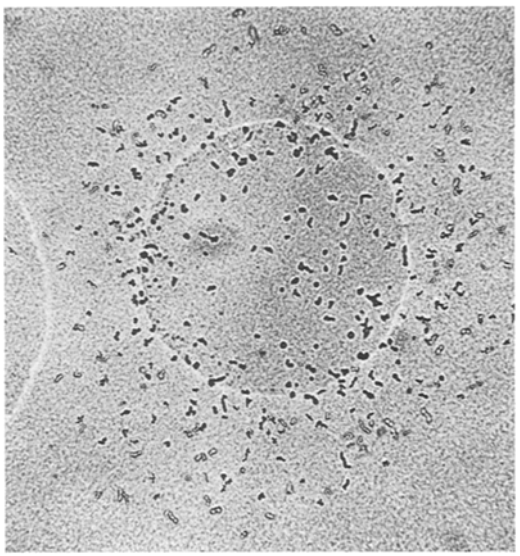

Fig. 2. High concentrations of the attractant deposited near the responding drop induce the myxamoebae to cross the margin of the drop on all sides. × 60.

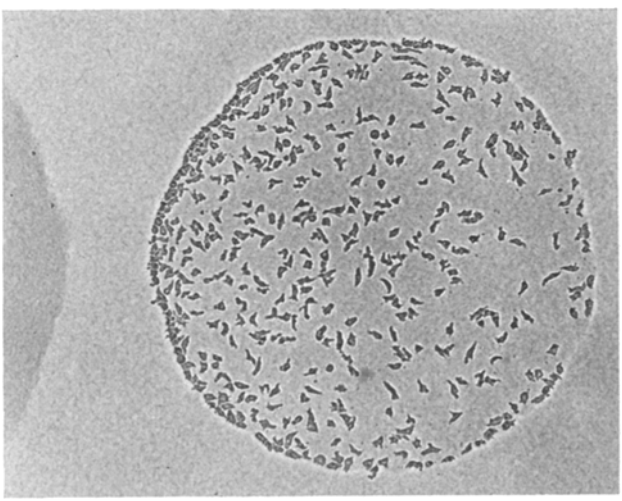

Fig. 3. Population of myxamoebae on washed non-nutrient agar of low rigidity. Drops (0.1 µl) containing 3×10^{-18} g cyclic AMP were deposited 3 times at 5 min intervals to the left of the responding drop. × 95.

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Therefore they probably play a minor role, if any, in the response of myxamoebae tested with active extracts.

Extracts of organs of higher organisms were also tested with this assay. Liver, spleen, heart, and kidney extracts were active. Urine and milk, even at dilutions of 1000 attracted myxamoebae. After purification of the extracts by column and paper chromatography[8], it was shown that cyclic 3′,5′-AMP was present. The attractants in human urine evoked a response similar to ca. 5 μM of cyclic 3′,5′-AMP. BUTCHER and SUTHERLAND[13] identified daily levels of 2–7 μM of cyclic 3′,5′-AMP in human urine. Consequently 3′,5′-AMP is the main, if not the only, attractant in urine. In collaboration with F. C. G. VAN DE VEERDONK, University of Utrecht, it was shown that extracts of dark pigmented skin of *Xenopus laevis* contained twice as much attractant as light pigmented skin. The activity of the attractant in 0.5 g darkened skin (wet wt.) was equivalent to ca. 1×10^{-8} g of cyclic 3′,5′-AMP[14]. SUTHERLAND and co-workers studied the production of cyclic AMP after the target cell was activated by a hormone. Interestingly enough I found that extracts of axolotl embryos without hormonal glands (blastula and neurula) are active. More detailed results will be published elsewhere[15].

Comparison of the chemotactic response induced by different concentrations of urine and cyclic 3′,5′-AMP

	Urine dilution × 500		3′,5′-AMP 0.01 μM		Urine dilution × 2000	
	Total positive	% positive	Total positive	% positive	Total positive	% positive
Test 1	25	83	32	73	14	48
Test 2	22	88	25	73	20	59
Test 3	18	60	22	47	7	23

The drops with attractant were placed 3 times with 5 min intervals near (less than 0.45 mm) the responding drops. Observation took place 5 min after the last deposition.

Zusammenfassung. Es wird ein mikrobiologisches Testverfahren beschrieben, mit dem sehr geringe Mengen von zyklischem Adenosin 3′,5′-monophosphat quantitativ nachgewiesen werden können.

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Hubrecht Laboratory, Uppsalalaan 1, Utrecht (The Netherlands), 17 November 1969.

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[15] I am grateful to Prof. K. B. RAPER in whose laboratory this assay was developed. I thank ROSEMARIE VAN DEN NOORT for her skilful assistance.

Blood Glucose and Liver Glycogen Content in Male Whirler Mice

Recent studies[1] with relatively small populations of homozygous male whirler versus heterozygous whirler mice have demonstrated marked but not significant reductions in plasma glucose levels (P 0.07), accompanied by significant decreases in liver glycogen. These data suggest differences in the carbohydrate metabolism and utilization processes of the neurological mutant. Previous investigations[2–5] of endocrine and metabolic differences between homozygous male and female whirler mice and their phenotypically 'normal' heterozygous littermates have indicated significant increases in adrenocortical activity and metabolism of the whirlers. The whirler mutation[6] represents a recessive behavioral and neurological factor located in the VIII linkage group. The mice are one of a group of waltzing recessive mutations[7] possessing an extremely nervous, restless and excitable nature. In addition to head-shaking and deafness, the mice display syndromes of rapid clockwise and/or counterclockwise circling locomotor activity. Neurological and labryinthine anomalies have been frequently associated with the waltzing syndrome[7]. The present paper sought to reaffirm and substantiate earlier impressions of hypoglycemia in the whirler mice by comparing groups with larger population sizes.

The original stock of male and female homozygous whirler and phenotypically 'normal' heterozygous mice were obtained from the Jackson Laboratory, Bar Harbor, Maine. After several generations of selective inbreeding, homozygous and heterozygous whirler male littermates were selected for study from matings of phenotypically 'normal' heterozygous females to whirler brother males. Genetically[7], the mutant whirler mice were homozygous for the recessive gene (wi wi). The heterozygous mice had the (wi+) genotype and appeared phenotypically 'normal' in behavior, locomotor activity and hearing. The mice were non-agouti and brown for coat color and hair pigment characteristics[7].

All animals were bred and raised in air-conditioned quarters with room temperatures maintained at 73–75 °F. When breeding units were capable of supplying sufficient numbers of mice for experimentation, homozygous and heterozygous male mice were weaned at 4 weeks of age for subsequent weekly body weight evaluations. The number of male mice was limited to 2 animals per cage to avoid crowding and fighting effects[8]. The cages were stainless steel, having dimensions of $6^1/_2 \times 10 \times 7$ inches and bedded with pine shavings.

At 16 weeks of age, the mice were sacrificed by rapid decapitation 1 h after body weight determinations to reduce undue stress and prolonged handling of the animals. Blood samples were collected in heparinized beakers for plasma glucose determinations[9]. All mice were autopsied rapidly for liver glycogen analyses by the procedure of

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