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Infection into paramecia of metagons derived from other mate-killer paramecia

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1. INTRODUCTION

In earlier papers we have described experiments showing that genic control of maintenance of the symbiotic mu (mate-killer) particles in *Paramecium aurelia*, stock 540 (syngen1), is mediated by the particulate elements we have designated 'metagons' (Gibson & Beale, 1962). In mate-killer cells containing either of the dominant genes M_1 and M_2, about a thousand metagons were considered to be present. When these genes were replaced by their recessive alleles and there was a period of subsequent growth, the metagons were thought to be distributed, without dividing, to the daughter cells until after 10–15 fissions cells containing only a single metagon were formed. Such cells were found to possess as large a complement of mu particles as cells with many metagons, and on division to yield one daughter cell containing many mu particles and one containing none.

Later it was shown that treatment of living paramecia with ribonuclease resulted in an inactivation or destruction of metagons, suggesting that RNA was an essential constituent (Gibson & Beale, 1963). However, since the concentrations of ribonuclease applied to the paramecia in these experiments were high (e.g. 0·5 mg./ml.) and the time of treatment long (6–12 hours), there was a possibility that the inactivating effect might be due to traces of other enzymes in the RNase samples, and in any case the effect of an enzyme on the metagons might be indirect. We have therefore now developed techniques for the extraction of metagons and their infection into fresh paramecia. The metagon-containing extracts can be subjected to various fractionating procedures and an attempt made to characterize the metagons more precisely. In this paper these techniques are described.

2. MATERIAL AND METHODS

(i) *General*

The various mate-killer and sensitive stocks of paramecia are the same as those used previously (Gibson & Beale, 1963), as are the basic methods for culturing the organisms.

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The following solutions were used:

Solution A

0·35 M sucrose
0·035 M $KHCO_3$
0·004 M $MgCl_2$
0·025 M KCl
1 mg./ml. bentonite
pH adjusted to 7·2 with 0·01 M KH_2PO_4.

Solution B

0·25 M sucrose
0·01 M KCl
0·01 M *tris*
0·005 M $MgCl_2$
1 mg./ml. bentonite
pH adjusted to 7·4 with 0·01 M KH_2PO_4.

Solution C

0·1 M $CaCl_2$
0·1 M sodium citrate
0·1 M NaH_2PO_4
0·1 M Na_2HPO_4

Solution D

0·01 M NaCl
0·001 M KCl
0·001 M $CaCl_2$
pH adjusted to 6·8 with KH_2PO_4

Solution E

0·1 M *tris*-HCl (pH 7·2)
0·001 M KH_2PO_4
5×10^{-3}M or 5×10^{-5}M $MgCl_2$

(ii) *Extraction of metagons from paramecia*

Extraction method 1a. The method of extracting metagons at present in use was carried out in the following manner: Mate-killer paramecia (stock 540) were grown at 28° C. in horizontally placed 3 l. Thompson bacteriological bottles (1 l. of medium per bottle), containing concentrated bacterized grass medium (using 6 g. dried grass/l.; pH adjusted to 6·8 with Na_2HPO_4). Usually a density of about 2500 animals per ml. was attained. The cultures were filtered through muslin, the paramecia then harvested by concentration in an Alfa-Laval continuous flow centrifuge and finally packed down by means of an oil-testing centrifuge. After concentration of the paramecia in this way they were still alive and the wet weight varied between 10 and 30 g. from 100 l. of the grass medium. About 10 g. wet weight was found most suitable for preparation of a batch of metagon extract. Two volumes of solution B, which contains bentonite, a powerful absorbent of ribonuclease (Brownhill *et al.*, 1959; Singer & Fraenkel-Conrat, 1961) was added to the living packed cells. Either of three homogenization procedures was then used to break up the cells: (i) freeze-thawing; (ii) grinding with a glass pestle, or (iii) shaking for 1 min. with 20% sodium lauryl sulphate.

The homogenate was centrifuged at 1500 g. for 10 min., 30,000 g. for 20 min. and 105,000 g. for 90 min. as shown in the diagram, thus yielding a sediment which we shall refer to as the 'microsome fraction' (see Fig. 1).

The 'microsomal fraction' was gently homogenized with a plastic homogenizer in 1 ml. solution E containing 5×10^{-3} M $MgCl_2$, the suspension was spun at 15,000 g. for ½ hour, and the supernatant thus obtained treated with 1 ml 1% sodium deoxycholate for 20 min. The latter procedure has been shown (Littlefield *et al.*, 1955; Pogo *et al.*, 1962) to eliminate membranes from the microsomes and to solubilize some protein. By spinning again at 105,000 g. for 90 min. a pellet was obtained which was considered to be composed mainly of ribosomes. It will be henceforward

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referred to as the 'ribosome fraction', though it must be pointed out that presence of ribosomes has not yet been confirmed by electron microscopy or analytical centrifugation.

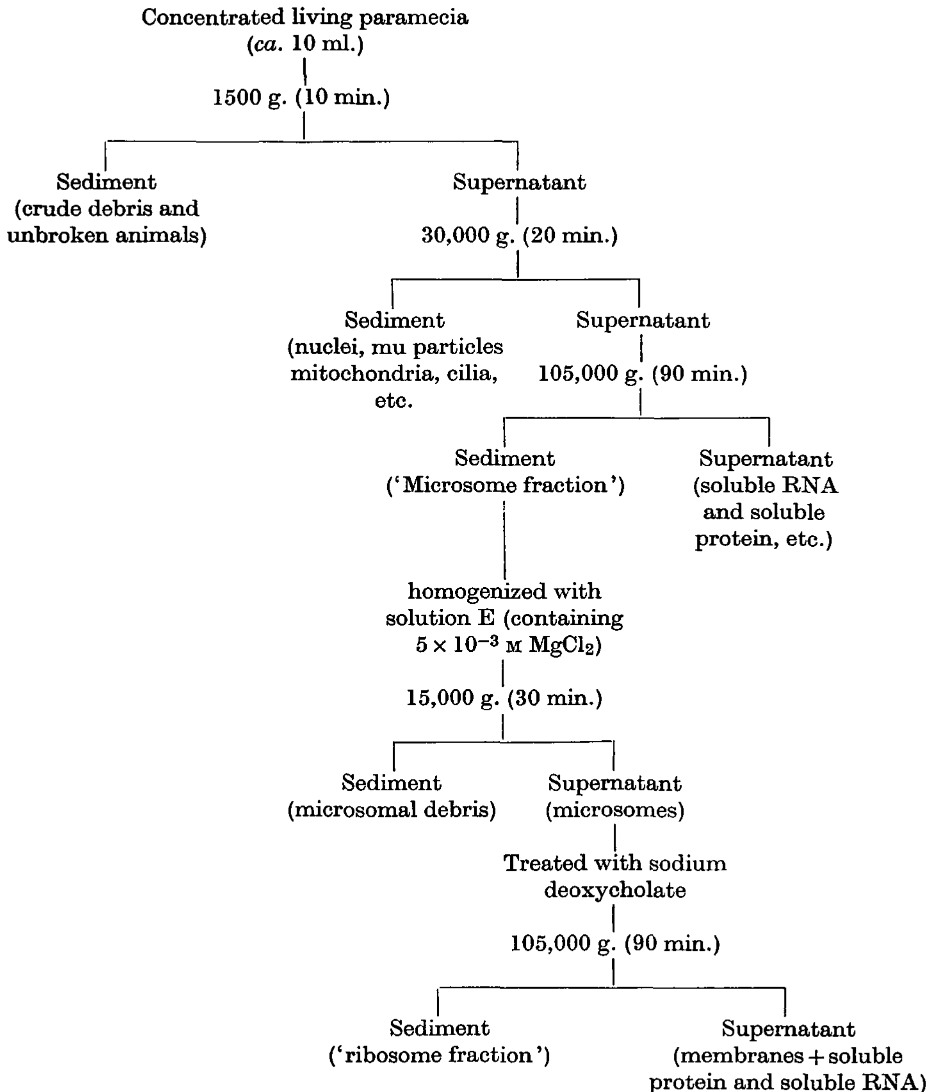

Fig. 1. Fractionation of paramecium homogenate by method 1 a. Identification of the constituents of the various fractions must be considered as tentative. See, however, Preer & Preer (1959).

The final ribosomal pellet was shaken up in solution E containing 5×10^{-3} M $MgCl_2$, and stirred with a glass rod. Aliquots were pipetted out and stored in a deep freeze at $-20°$ C. The high speed supernatants were also stored in this way. The whole procedure, subsequent to the initial homogenization, was carried out at about $+4°$ C.

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In addition to method 1a, the following variant methods to extract metagons were also successfully used at various times.

In *extraction method 1b*, about 4 g. (wet weight) of packed mate-killer paramecia were suspended in 8 ml. exhausted culture fluid with added bentonite (1 mg./ml.) and homogenized by forcing the concentrated organisms through a hypodermic syringe. After spinning at 5000 g. for 15 min., a sediment containing nuclei, etc., was obtained, and the supernatant was respun at 50,000 g. for 2 hours, producing a new sediment containing mitochondria and mu particles. The various sediments and supernatants were then tested for metagon activity.

Extraction method 1c involved homogenization by freeze-thawing, or by means of a glass pestle, in 2 vols. solution A. After spinning at 1500 g. (10 min.), 15,000 g. (15 min.), 30,000 g. (30 min.) and 105,000 g. (90 min.) a pellet was obtained and this was suspended in 2 vols. 0·25 M sucrose with a plastic homogenizer. After a further spinning at 15,000 g. a brown pellet ('dirt') was obtained. The final cloudy supernatant was considered to be the microsomal fraction, and was pipetted out in 0·5 ml. vols. and stored in Pyrex tubes at $-20°$ C. The 105,000 g. supernatant and low speed sediments were also stored at $-20°$ C.

(iii) *Detection of metagons in extracts*

The basic method to be described here consists in the infection of metagons into paramecia containing mu particles, as shown by the ability of daughter cells derived from those infected also to maintain mu particles. It is relevant here to recall that metagon activity cannot be detected in the absence of mu particles, that mu particles are not maintained in the absence of metagons, and that it has not yet been possible to infect mu particles into cells from the exterior medium. Hence, the following two types of recipient cells were used in tests with infecting fluids: (i) 'eleventh fission' animals of genotype $m_1m_1m_2m_2$, derived by autogamy from heterozygotes, and containing mu particles but only a small number (usually 1, 2 or 3) of metagons, as described earlier (Gibson & Beale, 1962) and (ii) animals of genotype $M_1M_1M_2M_2$ (stock 540), containing mu particles but no metagons, the latter having been previously destroyed by ribonuclease treatment (also described earlier, Gibson & Beale, 1963).

Infection into 'eleventh fission' mate-killer animals was carried out as follows. Groups of about twenty paramecia in 0·5 ml. solution C were added to 1 ml. samples of the metagon-containing solutions under test (diluted with solutions E, C, distilled water or exhausted culture fluid) and the paramecia allowed to swim in the mixture for 12 hours (in later experiments only 30 min.), at room temperature. Bentonite was added to give a concentration of 1 mg./ml. After the infection period the paramecia were taken out singly, washed in solution C containing 1 mg./ml. bentonite, then placed in bacterized culture fluid and allowed to divide three times. The groups of eight cells thus obtained were then starved in solution C for 12–24 hours, crushed and examined by phase-contrast microscopy for presence of mu particles.

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The basis of this method was our earlier finding (Gibson & Beale, 1962) that clones of 'eleventh fission' animals consist of 60% which lack mu particles entirely, and 40% which contain many mu particles but only a small number (usually 1, 2 or 3) of metagons. On division of the latter cells—which are the only ones of value in the present experiments—the progeny consist mainly of cells lacking mu particles but always a few containing them. The uptake of metagons into 'eleventh fission' cells would be expected to result in an increase in the proportion of mu-containing cells after a certain number of further fissions. With an appreciable uptake of metagons, all eight cells produced after three further fissions of the 'eleventh fission' animals would be expected to possess mu particles, instead of only one, two or three out of eight if there were no uptake. Uptake of metagons into any of the 60% sensitive 'eleventh fission' animals would not be detected by this method, since once the mu particles are lost from a cell, they are not re-formed even if metagons are present.

For the infection method (ii) using ribonuclease-treated $M_1M_1M_2M_2$ mate-killer animals as recipients the following procedure was adopted. About 2 g. (wet weight) of stock 540 animals (containing mu particles) in 2 ml. exhausted culture fluid (i.e. about 10^7 animals per ml.) were treated with an equal volume of a ribonuclease preparation (1 mg. bovine pancreatic ribonuclease in 1 ml. solution C or solution D), i.e. the final concentration of ribonuclease was 0·5 mg. per ml. (The RNase solution was briefly heated to boiling point before use.) The mixture of cells and enzyme was shaken intermittently for 6–12 hours at room temperature. There was usually an initial rapid effect on the animals, 90% being killed, though the effect of ribonuclease varied in different experiments for unknown reasons, and the concentration was adjusted when necessary to give about 90% death. The surviving paramecia were transferred several times through solution E containing bentonite, and then exposed in groups of twenty to the infecting metagon fractions in the same manner as described above for infection method (i). It was necessary to check the cells periodically since occasionally division occurred during the infection period. Dividing cells were discarded.

After treatment the paramecia were isolated singly into bacterized culture medium and allowed to divide at least once. (The actual number of divisions is irrelevant.) Samples of the daughter cells thus obtained were starved down in non-nutrient medium (solution C) for 24 hours, crushed and examined for presence of mu particles by phase-contrast microscopy.

This method is based on our earlier finding (Gibson & Beale, 1963), that ribonuclease-treatment of stock 540 mate-killer cells results in an immediate destruction of metagons, but persistence of the mu particles until the next fission after treatment. Between the first and second fissions following loss of metagons all mu particles disappear however. After the second fission, if the dominant genes M_1 or M_2 are present, more metagons, but no mu particles, are formed. If metagon infection takes place following ribonuclease-treatment but before the succeeding fission, mu particles will be maintained during the critical period between the first and second post-treatment fissions, and thereafter in all the progeny derived from such infected

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cells. If infection does not occur, none of the progeny will contain mu particles unless the ribonuclease treatment has failed to eliminate all the pre-existing metagons.

(iv) *Extraction of RNA from microsomal fractions*

In this procedure the microsomal sediment (20–50 mg.) prepared by extraction method 1a was suspended in 0·5 ml. solution E and heated with an equal volume of 1% sodium lauryl sulphate solution for 15 min. at 40° C. as described by Hall & Doty (1959). The solution was then placed in an ice-bath for 15 min. and all the subsequent steps were carried out at +4° C. An equal volume of 90% (w/v) phenol was added to the supernatant and the solution shaken for 10 min. by hand at +4° C., a procedure known to dissociate protein from nucleic acid. The solution was then centrifuged at 2000 g. for 5 min. The clear upper aqueous layer was drawn off with a pre-cooled pipette, and in some cases the interphase layer was also taken. As expected from previous work (Hall & Doty, 1959) the lower opaque layer contained the denatured protein, and the upper, aqueous layer contained nucleic acid. The latter fraction was extracted three times with phenol followed by centrifugation at 2000 g. for 10 min., and the final aqueous layer extracted three times with ether to remove any phenol still present. The ether layer was pipetted off each time and finally nitrogen was bubbled through the remaining aqueous solution to remove the last traces of ether. The resultant nucleic acid solution was dialysed overnight against solution E.

In some cases the nucleic acid solution was treated with two volumes cold absolute alcohol and left at +2° C. overnight. The white precipitate which formed was spun down at 4° C. and redissolved in solution E. Nitrogen was bubbled through the alcohol supernatant, producing a further nucleic acid precipitate, which was also redissolved in solution E.

(v) *Storage of fractions*

Various solutions were used for suspension of the microsome and nucleic acid preparations stored at −20° C., e.g. solution C, distilled water and 0·25 M sucrose, but metagon activity was retained longest (about 2½ months), when the materials were stored in *tris*-(0·1 M)-citrate (0·05 M) buffer at pH 6·8.

3. RESULTS

(i) *Metagon infection from fractions prepared by extraction method 1a*

In these experiments ribonuclease-treated stock 540 paramecia were used as the recipients. The microsome pellets prepared from various stocks by method 1a were suspended in 2 ml. solution E, diluted 1:100 in solution E, and 1 ml. portions then used in the infection tests. The 105,000 g. supernatant (undiluted) was also tested for infectivity, as were some of the earlier sediments (resuspended in 2 ml. solution

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E and diluted 1:100). The results are shown in Table 1. Infection of metagons was successful (i.e. into 90–95% recipient paramecia) from the 105,000 g. sediment ('microsome fraction') derived from any strain containing either of the dominant genes M_1 or M_2. The 105,000 g. supernatants (at full strength) also appeared to show a slight amount of infectivity. None of the lower speed sediments, nor any preparations derived from paramecia of genotype $m_1m_1m_2m_2$ (stock 513) yielded any infective metagons.

Table 1. *Infection into ribonuclease-treated paramecia of metagons from fractions prepared by extraction method 1a*

Stocks used for preparation of metagon extracts	Fractions tested for infection into ribonuclease-treated cells			
	1500 g. sediment	30,000 g. sediment	105,000 g. sediment	105,000 g. supernatant
Stock 540 (genotype $M_1M_1M_2M_2$)	0% (0) 42 treated	0% (0) 41 treated	90% (210)	10% (21)
Tester 5 (genotype $M_1M_1m_2m_2$)	Not tested	Not tested	90% (20)	10% (2)
Tester 7 (genotype $m_1m_1M_2M_2$)	Not tested	Not tested	95% (21)	15% (3)
Stock 513 (genotype $m_1m_1m_2m_2$)	Not tested	Not tested	0% (0) 31 treated	0% (0) 25 treated

The figures in the Table refer to the percentages and (in brackets) the actual numbers of paramecia infected with metagons. For details of dilutions of fractions, see text.

It should be pointed out that for preparation of metagon extracts, the mu particles need not be present in the cultures. In fact under the growth conditions used (concentrated grass medium) for preparation of dense cultures, the mu particles initially present had usually disappeared before the metagon extracts were made.

(ii) *Metagon infection from fractions prepared by extraction method 1b*

The various sediments prepared by method 1b were resuspended in 2 ml. and diluted 1:10, using solution C as diluent (stronger concentrations were toxic). They were then tested for ability to infect metagons into eleventh fission animals. The supernatants (undiluted) were also tested, and the results are shown in Table 2. The groups of eight cells formed by three fissions of the infected eleventh fission animals were classified as mate-killers (containing mu particles) or sensitives (lacking mu particles), and it was found that only treatment with the 50,000 g.

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supernatant, derived from stock 540 paramecia, produced a significant excess of mate-killer animals over the numbers in the groups of eight from untreated control animals. For example there were 21 (or 15%) groups of eight in which all the animals contained mu particles, after treatment with the 50,000 g supernatant, and only 2 (or 1%) in the controls. Extracts from stock 513 paramecia, as expected, did not raise the frequency of mate-killers in the animals tested over that in the controls. It is also of interest to note that the 5000 g. supernatant derived from stock 540 animals did not apparently give any infection by metagons, though the latter must have been present because infection was achieved with the 50,000 g. supernatant.

Table 2. *Infection into eleventh fission cells metagons extracted by method 1b*

Stocks used for preparation on metagon extracts	M:S	Fractions tested for infections into eleventh fission animals: Crude homogenate	5000 g. sediment (1/10 dilution)	5000 g. super-natant (undiluted)	50,000 g. sediment (1/10 dilution)	50,000 g. super-natant (undiluted)	Control: unin-fected cells
Stock 540	0:8		18	10	12	62	136
(genotype	1:7		5	3	7	3	41
$M_1M_1M_2M_2$)	2:6		4	6	6	6	36
	3:5	Toxic	6	2	1	8	31
	4:4		1	0	0	5	15
	5:3		0	0	0	9	10
	6:2		0	0	0	18	4
	7:1		0	0	0	20	2
	8:0		0	0	0	21	1
	Inc.		11	8	4	2	
Stock 513	0:8			8	12	25	
(genotype	1:7			3	4	6	
$m_1m_1m_2m_2$)	2:6			1	5	5	
	3:5		Not	1	1	7	
	4:4	Toxic	tested	0	1	3	
	5:3			0	0	1	
	6:2			0	0	0	
	7:1			0	0	0	
	8:0			0	0	0	
	Inc.			15	6	9	

The figures in the columns headed 'sediment' or supernatant' refer to the numbers of groups of eight cells classified as shown. For details of dilution of infective preparations, see Text. The column headed M:S (Mate-killer:Sensitive) indicates the classes of octets formed by three fissions of the eleventh fission cells which have been exposed to infection. Inc. = incompletely classified group, due to loss of some animals.

(iii) *Metagon infection from fractions prepared by extraction method 1c*

These fractions were used for infection tests into eleventh fission animals. Each of the sediments was resuspended in 2 ml. 0·25 M sucrose and diluted 1:10 with solution C. The supernatants were tested undiluted. The 105,000 g. sediment (microsome fraction) produced the most striking results, as shown in Table 3. There

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Table 3. *Infection into eleventh fission animals of metagons extracted by method 1c from stock 540*

Classification of animals after three fissions of infected eleventh fission cells		Fractions tested for infections into eleventh fission animals							
Mate-killers	Sensi-tives	1500 g. sediment	1500 g. supernatant	20,000 g. sediment	20,000 g. supernatant	30,000 g. sediment	30,000 g. supernatant	105,000 g. sediment	105,000 g. supernatant
0	8	10	8	9	5	10	16	52	45
1	7	3	2	3	2	3	4	0	10
2	6	4	3	1	1	5	3	0	8
3	5	3	2	3	1	1	6	1	7
4	4	0	0	1	0	1	1	1	6
5	3	0	0	1	0	0	1	6	3
6	2	0	0	0	0	0	0	10	1
7	1	0	0	0	0	0	0	11	2
8	0	0	0	0	0	0	0	31	4
Incompletely classified		8	4	7	3	1	8	20	15

For details of dilutions of infective preparations, see Text.

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were 52 groups of eight animals with none containing mu particles, and 60 groups with various numbers of animals containing mu particles. The former are presumed to have been derived from eleventh fission animals originally not containing any metagons, and the latter from eleventh fission animals originally containing a small number of metagons (The expected proportion of these two classes, based on earlier work (Beale & Gibson, 1962) is actually 67:45.) Amongst the second group, it will be noted that there were none in the 1:7 and 2:6 (mate-killer:sensitive) classes, and these were the commonest in the offspring of uninfected eleventh fission animals; but there were no fewer than 31 groups of eight consisting entirely of mate-killer animals, a class which is very rare in the offspring of uninfected eleventh fission animals. It is clear that eleventh fission animals originally containing only 1–3 metagons were infected by several more metagons, thus shifting the distribution of the frequencies of mate-killer and sensitive animals in the groups of eight. It is concluded therefore that treatment of eleventh fission animals with the resuspended 105,000 g. sediment at a dilution of 1:10 yielded metagon infection into 100% of recipient cells, many (if not all) of them receiving more than one metagon.

Table 3 also shows that the 105,000 g. supernatant was slightly infective, but none of the earlier supernatants were, even though they must have contained metagons.

(iv) *Metagon infection from the 'ribosome fraction'*

A 'ribosome fraction' was prepared by treating the 'microsome fraction' with sodium deoxycholate, thus removing the membranes. By centrifuging at 105,000 g. a ribosomal pellet and supernatant were obtained, starting with 20 mg. microsome

Table 4. *Comparison of infectivity of preparations before and after treatment of microsome pellet with sodium deoxycholate*

	Original microsome preparation		Ribosome preparation obtained by deoxycholate treatment	
	105,000 g. pellet suspended in 2 ml., diluted 1/1000	105,000 g. supernatant, undiluted	105,000 g. pellet suspended in 2 ml., diluted 1/1000	105,000 g. supernatant, diluted 1/50
Percentages and numbers of cells infected	50% (20)	8% (4)	100% (41)	5% (2)

pellet resuspended in 2 ml. solution. The ribosomal pellet was resuspended in 2 ml. solution E and diluted 1:1000 with solution E (containing 5×10^{-3} M $MgCl_2$), and the final supernatant was diluted 1:50 in solution E. These two solutions were then tested for ability to infect metagons into ribonuclease-treated stock 540 animals,

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and for comparison, similar infection tests done with a resuspended microsome pellet and microsome supernatant. The results are shown in Table 4, and indicate that the ribosome pellet apparently yields more infective metagon units than the original microsome pellet. The final (ribosome) supernatant also exhibits a slight amount of metagon infectivity, even at 1:50 dilution (though this is based on only two infected cells).

(v) *Metagon infection from the nucleic acid fraction*

Microsome pellets were resuspended and subjected to phenol extraction, as described in the methods section, and the nucleic acid fraction finally obtained tested for ability to infect metagons into ribonuclease-treated stock 540 paramecia. Such nucleic acid preparations were found to be highly infective, but the numerical data showing this are deferred to a later section (p. 98), dealing with the relation between concentration of metagons in solutions and the amount of infection.

By precipitating the nucleic acid fraction with alcohol, and subsequently dissolving the precipitate in solution E, an infective solution was also obtained, though only at high concentrations, i.e. precipitation resulted in loss of most of the metagon activity.

(vi) *Effect of variation in magnesium content on metagon infectivity of microsome fractions*

A microsome pellet was prepared as described previously and resuspended in 2 ml. solution E, containing 5×10^{-5} M $MgCl_2$, which we will refer to as a 'low magnesium' solution. After centrifuging at 15,000 g. for 10 min. to remove any debris the supernatant was centrifuged again at 105,000 g. for 1 hour. The final supernatant (undiluted) was tested for metagon infectivity* into ribonuclease-treated cells, as was also the pellet after resuspension in 2 ml. solution E containing 5×10^{-5} $MgCl_2$ and diluted 1:1000. The results are shown in Table 5a, and when compared with those in Table 4 indicate that in the 'low magnesium' medium, some of the infective material has become dissociated from the microsomes and is now present in the supernatant.

In a further experiment the microsome pellet was resuspended in 'low magnesium' medium and spun at 15,000 g. as above. The magnesium content of the suspension was then once again raised to 5×10^{-3} M by addition of $MgCl_2$, and the preparation spun at 105,000 g, after which the supernatant was drawn off, the sediment resuspended in 1 ml. solution E containing 5×10^{-5} M $MgCl_2$ and diluted 1:1000. Tests were then made of the infectivity of the final sediment and supernatant and the results are shown in Table 5b. They show that restoration of the suspension to the 'high magnesium' level resulted in a re-association of most of the metagon activity with the microsomes.

* In all infection tests from here on the period of exposure of the animals to the infecting fluid was 30 min., not 12 hours as previously (except where otherwise stated).

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Table 5. *Effect of variation in magnesium concentration on infectivity of microsome pellet and supernatant*

	(a) Microsome preparation suspended in 'low Mg' medium and re-centrifuged		(b) Microsome preparation in 'low Mg' medium, returned to 'high Mg' and re-centrifuged	
	105,000 g. pellet in 2 ml., diluted 1/1000	105,000 g. supernatant undiluted	105,000 g. pellet in 2 ml., diluted 1/1000	105,000 g. supernatant, undiluted
Percentages and numbers of cells infected	10% (5)	60% (21)	45% (18)	4% (4)

The effect of resuspension at 'high' and 'low' magnesium concentrations on infection with the metagons from a given microsome pellet was also studied. A few data of this sort are shown in Table 6, from which it appears that suspension of microsomal material in the 'low magnesium' solution results in some increase (about two-fold) in number of infective metagon units.

Table 6. *Effect of variation in magnesium concentration on infectivity of material from a given microsome pellet* ca. *30 mg.* (*net wt.*)

Dilution of infecting solution	Resuspension in 'high Mg' solution ribonuclease-treated cells			Resuspension in 'low Mg' solution ribonuclease-treated cells		
	Infected	Not infected	Dead	Infected	Not infected	Dead
1/500	12	8	2	12	0	5
1/1000	10	10	2	8	4	4
1/2000	6	14	0	6	4	1
1/5000	1	19	2	3	17	1

(vii) *Relation between concentration of metagons and infectivity*

The effect of varying the concentration of metagons derived from microsomal, ribosomal and nucleic acid preparations was studied in detail, and the data are collected in Table 7. Each of the three preparations was derived from approximately the same wet weight of original material, yielding about 30 mg. of the microsome preparation, and *ca.* 10 mg. of the ribosome preparation. Each of the three types of preparation was resuspended in 2 ml. 'high magnesium' solution, and a series of

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Table 7. *Effect of varying concentrations of microsome, ribosome and nucleic acid preparations on proportion of ribonuclease-treated cells infected with metagons*

	Microsome preparation			Ribosome preparation			Nucleic acid preparation		
Dilution	Infected	Not Infected	Dead	Infected	Not Infected	Dead	Infected	Not Infected	Dead
Undiluted	0	0	100%	0	0	100%	0	0	100%
1/2	0	0	100%	0	0	100%	24	0	
1/10	0	0	100%	0	0	100%	21	0	
1/200	15	5	25%	10	0	50%	20	0	
1/500	12	8	1%	18	2	10%	20	0	
1/1000	→10	10	1%	16	4	5%	20	0	
1/2000	6	14	0%	→10	10	10%	18	2	
1/3000	4	16	0%	6	14	1%	—	—	
1/4000	2	18	0%	3	17	0%	11	9	
1/5000	1	19	1%	1	19	5%	→9	11	
1/6000	1	19	5%	0	20	0%	—	—	
1/8000	0	20	5%	0	20	10%	—	—	
1/10,000	0	20	5%	0	20	5%	4	16	
1/20,000	0	20	5%	0	20	0%	—	—	

→ = concentration giving infection of approximately 50% of the cells.

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dilutions made. A small number (*ca.* 25) ribonuclease-treated stock 540 paramecia was placed in 1 ml. of each of the diluted samples for half an hour at room temperature. A convenient measure of the metagon activity of a solution is the concentration which gives infection of 50% of the cells under these conditions. Thus, the microsomal fraction gave such a result at dilution 1:1000, the ribosomal at 1:2000, and the nucleic acid at 1:4–5,000. As for the relation between concentration of metagons in a solution and percentage of cells infected in 30 min., the data are insufficient for any exact formulation, but there is an approximately linear relation with the dilution of the microsome preparation. With infection from the ribosome preparation, the proportion of infected cells seems to fall off more rapidly with decreasing metagon concentrations. In considering these results, complications due to instability of the metagons and 'clumping', etc., as described below, should be borne in mind.

(viii) *Rate of uptake of metagons into paramecia from infective fluids*

In this section we report the results of infection tests by exposure of ribonuclease-treated stock 540 animals to microsome and nucleic acid preparations for times ranging from 5 min. to 48 hours at 20° C. The results are shown in Table 8. It will

Table 8. *Infection of metagons into ribonuclease-treated stock 540 paramecia following exposure for various periods of time to infecting fluids at 20° C*

	Microsome preparation			Nucleic acid preparation		
Time of exposure to infecting fluid	Infected	Not infected	Dead	Infected	Not infected	Dead
5 min.	5	74	1	6	73	1
15 ,,	15	65	0	18	62	0
30 ,,	35	45	0	31	46	3
1 hour	30	46	4	39	39	2
2 hours	26	49	5	45	35	0
3 ,,	19	59	2	60	19	1
6 ,,	12	67	1	61	15	4
12 ,,	5	75	0	68	10	2
24 ,,	1	74	5	69	6	5
48 ,,	0	74	6	72	2	6

The preparations were diluted so as to give infection of approximately 50% of the cells after 30 min.—i.e. 1/1000 of the microsomal pellet suspended in 2 ml. and 1/5000 of the nucleic acid material in 2 ml.

be seen that maximum infection from the microsome preparation occurred in half an hour. Longer exposure to the infecting fluid apparently resulted in infection of a smaller percentage of cells, suggesting some kind of destruction of metagons already inside. With the nucleic acid preparation, however, the proportions of cells infected rose steadily with increasing periods of treatment.

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With the aim of studying further the curious inactivation effect of metagons introduced into the cells from microsome preparations, the effect of starvation immediately after infection for half an hour was studied, since prolonged exposure to the infecting fluid caused starvation of the animals and this might be the cause of the inactivation of metagons already infected. The results are shown in Table 9, from which it is clear that approximately 50% of the cells were infected following half an hour's exposure to the microsome preparation, no matter whether there was a subsequent period of up to 12 hours' starvation or not.

Table 9. *Effect of starvation following infection with microsome pellet material into ribonuclease-treated cells*

Period of post-infection starvation	Cells	
	Infected	Not infected
1 hour	11	9
2 hours	9	11
4 ,,	7	13
6 ,,	9	11
8 ,,	10	10
10 ,,	11	9
12 ,,	9	11

Period of infection 30 min. Concentration of the preparation as in previous experiment.

In a further experiment a group of 50 cells was exposed to infection by metagons in 1 ml. of a microsome preparation (diluted 1/1000 with solution E (high magnesium)) for half an hour at 20° C., washed in solution D, and then placed in a fresh sample of the microsome preparation for a second half-hour infection period. The

Table 10. *Infection of metagons by two successive treatments with microsome pellet material*

Treatment	Cells	
	Infected	Not infected
One ½ hour infection	11	9
Two successive ½ hour infections in separate solutions	18	2

Concentration of infective preparations as in previous experiments.

cells were washed in solution D, transferred to bacterized medium, allowed to divide once, then starved in solution C and scored for mu particles. The results are given in Table 10, and show that the second treatment significantly raised the proportion of cells infected. It would appear therefore that when cells are left for longer than

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half an hour in contact with the microsomal preparation, a destructive factor (possibly ribonuclease) develops and then passes into the animals, inactivating some of the metagons which had entered earlier.

(ix) *The effect of very weak ribonuclease treatment on metagons introduced into paramecia by infection*

Paramecia were exposed for half an hour to microsome and nucleic acid preparations known to be capable of infecting 50% of cells with metagons (i.e. dilutions 1/1000 and 1/5000 respectively). The recipients in this experiment were 'third fission' animals (i.e. of genotype $m_1m_1m_2m_2$ but containing mu particles and many metagons) and treated with ribonuclease (0·5 mg./ml. for 12 hours) to eliminate the metagons. (Thus, this is a combination of infection methods (i) and (ii), see above p. 88.) After infection, some of the 'third fission' animals were washed in solution C and treated with 1 ml. of very weak ribonuclease solution (5 μg./ml. in solution C for 1 hour). The animals were then washed once more in solution C (which contains bentonite), and the survivors allowed to divide three times in bacterized culture medium. In control tests, 'third fission' animals were exposed to the same infecting fluids but not subsequently given the weak ribonuclease treatment. The results are shown in Table 11.

Table 11. *Infection of metagons into 'third fission' paramecia (genotype* $m_1m_1m_2m_2$*), treated before infection with 0·5 mg. ribonuclease for 12 hours, and after infection with 5 μg. ribonuclease for 1 hour*

		Proportions of mate-killer and sensitive animals in groups of eight obtained by three fissions of treated cells			
		Infection with microsome preparation		Infection with nucleic acid preparation	
Mate-killer	Sensitive	Cells given weak post-infection ribonuclease treatment	No post-infection treatment	Cells given weak post-infection ribonuclease treatment	No post-infection treatment
0	8	6	7	8	5
1	7	0	4	0	0
2	6	1	2	0	0
3	5	0	1	0	0
4	4	0	0	0	0
5	3	0	0	0	0
6	2	2	0	0	0
7	1	1	0	4	2
8	0	3	0	2	2
Incompletely classified		3	3	4	2

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These show that where there was no subsequent weak ribonuclease treatment the microsomal preparation gave a total infection of 7 out of 14 cells, and the seven infected contained 1, 2 or 3 infective units, in the proportions of 4:2:1 respectively. When the infected cells were subsequently exposed to weak ribonuclease treatment, there were 7 out of 13 cells infected, but of those infected most appeared to contain at least 6–8 infective metagon units. When infection was from the nucleic acid preparation there was no difference in the proportions of mate-killer cells amongst the progeny of the third fission cells treated or not treated with weak ribonuclease. However, here all the animals which were infected yielded 7 or 8 mate-killer cells after 3 further fissions. It should be added that third fission cells treated with 0·5 mg./ml. ribonuclease for 12 hours but not subsequently exposed to the infective fluids gave 100% sensitive animals, and third fission cells not treated at all gave, after 3 fissions, 100% mate-killers.

In view of the small numbers and the complexity of the situation, these results cannot be interpreted with certainty at present. As a tentative explanation we propose that in both the microsome and nucleic acid preparations the metagons are in clumps. When the microsome preparation is used for infection, the clumps of

Table 12. *Effect of temperature on inactivation of metagon activity in microsomal and nucleic acid preparations*

Infective preparation	Time stored at temperature stated (hours)	Temperature of storage of infective preparation			
		4° C.	12° C.	20° C.	28° C.
Microsomal (1/1000)	1	12	8 (12)	10	1 (13)
	2	13	6 (11)	8	2 (10)
	3	11	7	9	0 (11)
	6	12	8 (9)	1	0 (9)
	8	11	5 (10)	1	0 (12)
	15	8	2 (12)	0	0 (12)
	18	8	2 (10)	0	0
	20	6	1 (9)	0	0 (11)
	24	7	1 (11)	0	0 (12)
	48	7	0 (9)	0	0 (8)
	72	4	0 (10)	0	0 (5)
Nucleic acid (1/5000)	1	8	12	10	10 (10)
	2	10	12	12	9 (11)
	8	12	15	13	6 (12)
	15	15	14	11	5 (11)
	24	12	14	10	1 (10)
	48	8	12	8	0 (8)

The data in this Table refer to the numbers of paramecia infected out of twenty treated.

The figures in brackets are the numbers obtained when bentonite was added to the preparations kept for the stated times at the various temperatures.

These preparations were diluted with 'high Mg' medium.

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infected metagons are broken up after infection by the weak ribonuclease treatment, and when the nucleic acid preparation is used for infection, the clumps break up inside the cells spontaneously. Obviously, however, these matters demand further investigation.

(x) *Inactivation of metagons in extracts at different temperatures*

The effect of temperature on the rate of inactivation of metagons in microsome and nucleic acid preparations at dilutions known to be capable of yielding initially 50% infection was studied by placing samples of the solutions for various times at 4°, 12°, 20° and 25° C., and then adding groups of 20 ribonuclease-treated stock 540 paramecia to the samples for half an hour at 20° C. to test for the ability of the latter to give infection. Part of the experiments were duplicated, in one set bentonite being present in the samples exposed to the various temperatures and in the other bentonite being absent. The results are shown in Table 12. These show that the microsome preparation is less stable than the nucleic acid preparation, but that the inactivation of metagons in the microsome preparation can be considerably reduced

Table 13. *Effect of ribonuclease and deoxyribonuclease on infectivity of metagon preparations*

Infective preparation	Enzyme		Buffer	Numbers of cells infected out of twenty treated
Microsomal (1/1000)	RNase:	0·5 mg./ml.	Soln. E (5×10^{-3} M $MgCl_2$)	12
		0·125 mg./ml.	,, ,, ,,	9
		0·5 mg./ml.	Soln. E (5×10^{-5} M $MgCl_2$)	0
		0·125 mg./ml.	,, ,, ,,	0
		5 μg./ml.	,, ,, ,,	0
	DNase:	0·5 mg./ml.	Soln. E (5×10^{-5} M $MgCl_2$)	11
		0·125 mg./ml.	,, ,, ,,	10
	Control		Soln. E (5×10^{-3} M $MgCl_2$)	12
			,, (5×10^{-5} M $MgCl_2$)	13
Nucleic acid (1/5000)	RNase:	0·5 mg./ml.	Soln. E (5×10^{-3} M $MgCl_2$)	0
		0·125 mg./ml.	,, ,, ,,	0
		5 μg./ml.	,, ,, ,,	0
		0·5 mg./ml.	Soln. E (5×10^{-5} M $MgCl_2$)	0
		0·125 mg./ml.	,, ,, ,,	0
		5 μg./ml.	,, ,, ,,	0
	DNase:	0·5 mg./ml.	Soln. E (5×10^{-5} M $MgCl_2$)	11
		0·125 mg./ml.	,, ,, ,,	12
	Control		Soln. E (5×10^{-3} M $MgCl_2$)	14
			,, (5×10^{-5} M $MgCl_2$)	10

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by addition of bentonite. The nucleic acid preparation is not appreciably inactivated in 48 hours at temperatures up to 20° C., but at 28° shows a steady loss of activity which can be prevented by addition of bentonite.

We tentatively conclude that inactivation is due to traces of ribonuclease present especially in the microsome preparation.

(xi) *Effect of ribonuclease and deoxyribonuclease on the metagon activity of the microsome and nucleic acid preparations*

Microsome and nucleic acid preparations capable of yielding about 50% infection of ribonuclease-treated cells were treated with the enzymes for 1 hour at 4° C. at pH 7·4, and the solutions were then tested for ability to infect cells with metagons. The results are shown in Table 13. Deoxyribonuclease had no effect on the metagon infectivity of the preparations, but ribonuclease destroyed all metagon activity at a concentration of 0·125 mg./ml. with the microsome preparation, and at 5 μg./ml. with the nucleic acid preparation. In these experiments the ribonuclease solutions were briefly heated to 100° C. before use.

These results show that the microsome preparations are more susceptible to the action of ribonuclease when suspended in 'low magnesium' medium than 'high', and further, that the metagons in the nucleic acid preparation are very much more susceptible to the action of ribonuclease than those in the microsome preparations, or, even more, those in the living animals (for data on the latter, see Gibson & Beale, 1963).

4. DISCUSSION

The results given above show that metagons derived from homogenates of paramecia containing the dominant genes M_1 or M_2 (or both) may be infected into other paramecia, and once inside the recipient cell exercise the characteristic function of maintaining the mu particles. Neither the method of homogenization, nor the composition of the homogenizing medium seems critical for preservation of metagons, except that bentonite, an inhibitor of ribonuclease, should be present initially. After removal of crude debris by slow speed centrifugation, however, it is unnecessary to have bentonite present during subsequent fractionation procedures.

The metagon activity remains in the supernatant after centrifuging at 50,000 g. for 120 min. but most of the activity is found in the sediment after centrifuging for 90 min., at 105,000 g., to produce the 'microsome' fraction. Metagon activity is retained—probably increased, in terms of numbers of infective units—following removal of the microsomal membranes by sodium deoxycholate treatment, producing the 'ribosome' fraction. Phenol extraction results in the retention of the metagon activity in the nucleic acid fraction (RNA), and again there appears to be an increase (approximately five-fold) in number of infective units, by comparison with those in the 'microsome fraction'. Metagon activity in extracts is destroyed by treatment with ribonuclease, very low concentrations (5 μg./ml. or less) being sufficient

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for this purpose if the nucleic acid fraction is treated. Metagon activity is retained for $2\frac{1}{2}$ months when the preparations are kept at $-20°$ C. in *tris*-citrate buffer, but gradually declines. Above freezing point metagon activity of solutions declines fairly rapidly, though in a nucleic acid fraction there is no detectible diminution in activity over a period of 48 hours at 20° C. or lower. With the microsome preparations metagon activity declines in a few hours at 12° or 20° C., but this can be prevented by adding bentonite to the preparations. Probably the liberation of traces of ribonuclease from the microsomes is the cause of this inactivation.

Both methods which have been used for testing the metagon activity of extracts by infection have given positive results, but each method has characteristic disadvantages. Infection into ribonuclease-treated stock 540 paramecia is a useful method for rapidly testing a number of different preparations. It does not, however, permit one to determine whether infection of a given animal is by a single metagon or several. However, an estimate of the activity of a preparation may be gained with this method by determining the concentration capable of infecting 50% of recipient cells with one or more metagons. The elimination of pre-existing metagons by treatment of the cells with ribonuclease is rather variable: too weak a treatment does not eliminate all the metagons, and too strong a treatment kills all the animals. It is therefore necessary to include ribonuclease-treated controls, without subsequent infection, in any critical experiment.

Infection into 'eleventh fission' ($m_1m_1m_2m_2$) animals has the advantage of enabling one to gain an idea of how many metagons have entered a cell, and does not suffer from the hazards resulting from the ribonuclease treatment. It is, however, very laborious to prepare a sufficiently large number of eleventh fission animals, and as many of the latter (60%) contain no mu particles they are useless for testing for metagon infectivity.

We do not know much about the actual infection process. Presumably it takes place via the alimentary apparatus of the paramecia, but if so it is remarkable that the digestive system does not contain ribonuclease capable of destroying the infecting metagons. In our early tests we included bentonite in solutions before and during the infection process, but this was discontinued when it was found to be unnecessary.

As for the nature of the metagon, present indications are that it is some kind of 'messenger' or 'informational' RNA. The findings that metagon activity is associated with a ribosome fraction, can be dissociated from the ribosomes by lowering the magnesium concentration, is preserved following removal of protein from the nucleoprotein, and that metagon activity of the nucleic acid fraction is extremely sensitive to ribonuclease—all favour such a view. Certainly the metagon is a structure bearing a genetic 'message', though whether its action involves synthesis of a specific protein is unknown. However, a very distinctive property of the metagon is its great stability. In our earlier work (Gibson & Beale, 1962) we showed that *in vivo*, metagons remained active for at least 18 cell generations, possibly for much longer. During all this time—or at least once during each cell cycle—the metagons must produce some factor necessary for the maintenance and reproduction of large numbers of mu particles.

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As is well known, the original discovery of messenger RNA in bacteria (Brenner *et al.*, 1961; Gros *et al.*, 1961) was of an extremely unstable element, but later work, especially that dealing with mammalian haemoglobin synthesis, indicates that some kinds of messenger RNA may persist and function for an appreciable time (for example, see Marks *et al.*, 1962). The metagon appears to be an example of a very stable 'messenger'.

Our present results suggest that the metagons are often aggregated in clumps, each unit of which is able to function on its own. In our earlier work (Gibson & Beale, 1962) there was also evidence for the non-random redistribution of metagons among dividing cells, though Reeve & Ross (1963) by a mathematical analysis, did not find that the data could be accommodated with any simple clumping hypothesis. This matter will be pursued in further studies.

A study of the RNA fraction containing metagon activity by electrophoresis on cellulose acetate paper is reported in another paper (Gibson, 1964).

SUMMARY

1. It has been shown that metagons, gene-controlled units necessary for maintenance of mu particles in *Paramecium aurelia*, can be extracted from cells containing one or both of the dominant genes M_1 and M_2, and reinfected into other paramecia. Provided the latter contain mu particles before infection, the introduced metagons resume their normal function of maintaining the mu particles.

2. Homogenates containing metagons have been fractionated in various ways. The metagon activity is found in the sediment after centrifuging at 105,000 g. for 90 min., and is retained after deoxycholate treatment removing the microsomal membranes, and after phenol extraction removing the ribosomal protein. By lowering the magnesium concentration, some metagon activity passes from the microsomes into the supernatant.

3. Metagon activity in RNA fractions is destroyed by ribonuclease at a concentration of 5 μg. per ml.

4. The metagons both in extracts and inside the cells appear often to be aggregated in clumps, the individual units of which are each functionally active in maintaining mu particles in living cells.

5. It is concluded that the metagon may be considered as a very stable and repeatedly functioning form of 'messenger' RNA.

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