Size and Conjugation in *Blepharisma.*

By

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With 1 figure in the text and plate 8.

Previous studies on conjugation of a number of races of *Paramecium multimicronucleatum* have indicated that a sudden decline in the food is the environmental factor most closely correlated with conjugation (GIESE, 1935 and 1938 c), which is in agreement with the results of many other investigations on various species of protozoa though not in agreement with those of others. It is also well known that preceding conjugation there is a decrease in size and that conjugants are smaller than non-conjugants in the same culture (PEARL, 1907; JENNINGS, 1911; WATTERS, 1912; CALKINS, 1912). Must animals subjected to a sudden decline in nutrition (1) reach a given size under a given set of environmental conditions before they will conjugate, or (2) is the size variable and dependent upon the particular size preceding the sudden change in nutrition? The following paper reports experiments attempting a decision between the two.

Materials and Methods.

Blepharisma undulans STEIN, isolated from local pond water in the autumn of 1933, was grown in hay infusion until April, 1934. Then one individual was washed free of bacteria and grown under the "standard conditions" reported in a previous paper (GIESE, 1938 a), viz., on a single strain of bacteria (*Pseudomonas ovalis*) in buffered (7.0) lettuce infusion (0.05 %) at 26 ± 0.1^{0} C.

The strain of Blepharisma used had the typical beaded macronucleus (as described by CALKINS, 1912) which condensed during division and during conjugation. Micronuclei were many but variable in number

and were present along but outside the macronucleus. The undulating membrane was large and typical. The race was very healthy though the division rate was low (1.4 per day at 26° C) and exconjugants showed at least 75 % viability (40 trials). This high survival was also found by STOLTE (1924) but stands in contrast to the low viability of conjugants of this species observed by CALKINS (1912).

The inorganic balanced salt solution referred to in the text is that previously described (GIESE, 1938 b) and the methods employed in washing the animals were similar to those already outlined (GIESE and TAYLOR, 1935). Bacteriological precautions were observed with cultures and experiments were performed in sterile Columbia watch glasses or in 1" syracuse watch glasses held in moist chambers and kept in an incubator at $26 \pm 1.5°$ C.

In all cases at least 9 experiments generally on three different days were performed to test each point. In many cases about 30 experiments were performed. Measurements were made with a camera lucida and stage micrometer and each mean reported is the average of at least 25 cases unless otherwise stated. For immediate observations animals were quickly injected into methyl green or DAFANO'S fixative; for permanent mounts SCHAUDINN'S fixative and the FEULGEN reaction were used. Photographs of actively moving animals were made with the apparatus previously described (GIESE, 1938 b). All the photographs were taken with the same magnification and were printed to the same enlargement and are therefore directly comparable.

Experimental.

SONNEBORN (1937) has demonstrated in *Paramecium aurelia* the presence of two "races" (or sexes), progeny of neither of which conjugate among themselves but which conjugate with one another when the two are mixed. Since it is possible that any protozoan might show the same phenomenon, tests were made to determine whether the two such "races" were necessary for conjugation in the clone of *Blepharisma* used here.

Individuals were isolated in tubes 7×4 mm. and grown in the tubes in the usual medium in the usual way. On the third day after transfer animals were inoculated one each into a series of 5—15 tubes, the inoculum being chosen from one of the tubes showing a flourishing culture. Ten such series, a total of 75 trials, were made. Examinations were made every 12 hours for conjugation. In every series and in every tube of each series conjugation was found

to occur. Conjugants generally appeared on the 5th to the 7th day after transfer, the number present varying from a few per cent to as many as 63 % of the population of the tube counted in one case.

SONNEBORN (1937) found that in *P. aurelia* segregation of the sexes occurs after endomixis. Endomixis has not been described for *Blepharisma*, and DAWSON (1928) who investigated the vitality of *B. undulans* for 4 years (one clone throughout the period, 3 clones part of the time), staining every few days for the entire period and daily for a period of two months, never saw signs of endomixis. To determine whether endomixis were occurring in the clone here used, samples were stained with acetocarmine every 3rd or 4th day in series 6 to 9. In series 10 samples were stained daily. No endomixis was observed. Also in many permanent slides of vegetative individuals made at various times with FUELGEN's reaction endomixis was not seen.

The results indicate that either two distinct types are not necessary for conjugation in this clone of *Blepharisma* under the conditions of the experiments, a situation SONNEBORN (1937) found to obtain for some of his races of *P. aurelia*, or segregation into two sexes occurs after each division, in which case equal numbers of the sexes would be present at any given time. The following studies on the effects of the environment would not be vitiated by disproportions of conjugating types in the population.

In "standard" cultures of *Blepharisma* in the 15 cc. tubes conjugants are observed in small numbers subsequent to the clearing of the bacterial opacity (5th day) and a decrease in size of the animals. The numbers of conjugants appearing in standard cultures is always small, being at any given time not more than a few per cent of the population.

To determine whether blepharismas would conjugate when the food supply was suddenly diminished, animals from 5th day cultures were washed free of organic materials in the unbuffered inorganic salt solution, concentrated tenfold, and placed in the incubator at 26° C. In such cultures epidemics in which from 60 to 70 % of the animals were conjugating occurred about 24 hours after setting out.

An epidemic of conjugation lasts for about 48 hours, although individual conjugants generally remain united for a much shorter time — approximately 24 hours. At the timeare a maximum number in conjugation some have just united, others are beginning to exconjugate. The course of an epidemic is plotted in Text.-Fig. 1. The data from one series of 10 cultures and another of 7 cultures are estimates since counts could not be made without picking the

animals in a pipette and so disturbing the course of the epidemic. Even if only approximate, they indicate the trend of events.

Conjugation seems to occur when there is a decrease in food occurring within a certain lapse of time. When the change occurs over a greater time lapse one might expect conjugation to occur only in smaller numbers of individuals or not at all. If there is a positive nutritive change one should not expect conjugation to occur. To test these ideas the following experiments were performed.

First, animals from standard cultures 8, 9, 10 and 16 days after subculture were washed and concentrated in the usual way and observed for conjugation. Epidemics occurred sporadically in the first group, more irregularly and with fewer conjugating in the next two and not at all in the last. It appears that in the standard cultures starvation occurs slowly and the change to the lower nutritive level occurs too slowly to evoke an epidemic. Such animals have reached a lower nutritional level and when they are now washed and concentrated, the differential achieved, though negative, is no longer great enough to evoke conjugation. If the animals fail to conjugate because the change is not sufficient, and not because of some time in the cycle, refeeding them should restore a high nutritional level and conjugation should then follow a sudden decline. To test this possibility animals from cultures 8 and 16 days after inoculation were washed free of the original culture medium and concentrated tenfold in the first and twentyfold in the second case and each fed 5 drops of the "standard" suspension of bacteria. This food was quickly taken up and approximately 36—48 hours after feeding epidemics of conjugation occurred in both cases. The "standard" suspension of bacteria was made by suspending a mm. loopful of *Pseudomonas ovalis* (grown at 26° C on 2% agar — 2% yeast extract and used 24 hours after streaking) in 1 cc. of the balanced salt solution. The

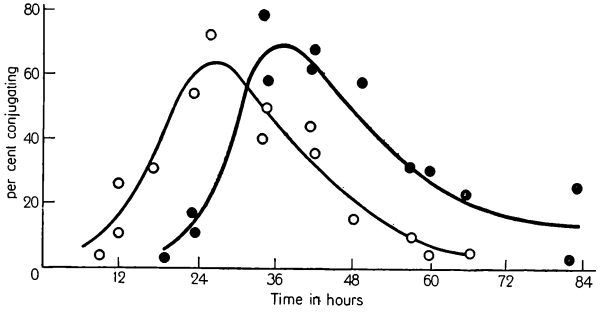


Fig. 1. Open circles, unfed cultures, animals from 5 cc. of a 5-day old culture concentrated, after washing, into $^1/_2$ cc. inorganic salt medium; closed circles, cultures treated as above but fed $7^1/_2$ drops standard suspension of bacteria. Data are from estimates.

borders of the loop were scraped even with the edge so the volume of bacteria was that of a cylindroid of the inner dimensions of the loop.

To test the effect of a small increase in nutritional level, suspensions of bacteria were added to the suspensions of animals from standard cultures washed (5th day) in balanced medium. Such additions of food should at least temporarily inhibit the onset of the epidemic, the delay being proportional to the food added. Experiments showed that this was the case. One drop of such a suspension added to the washed blepharismas from 5 cc. of a standard culture concentrated into $^1/_2$ cc. of the balanced salt solution did not appreciably delay the epidemic, but $7^1/_2$ drops produced a delay of about 12 to 16 hours. Estimates from two series of experiments are plotted in Text.-Fig. 1. While a proportionality was always observed, the technique has not been refined to the point where an exact delay can be predicted. In general it may be concluded that feeding delays the onset of the epidemic of conjugation here as in *Paramecium*.

It was observed that while epidemics occurred after a delay in cultures so fed, at times more animals were in conjugation at the maximum than in the controls. This seems paradoxical, but it may be interpreted as resulting from the greater uniformity in nutritional state induced by a sparce but evenly distributed feeding followed by a rapid exhaustion of the food. In controls the decline in nutritive state of some individuals may have already occurred and the differential achieved by washing was insufficient to induce conjugation in as many individuals. The various experiments outlined above are apparently interpretable on a similar basis.

A change in nutrition results in a corresponding change in size, the exact magnitude of the change in size depending on the nature of the relationship between nutrition and size as well as upon the environmental conditions. Without change in other factors a decrease in nutrition results in reduction of size. The amount of reduction in size preceding conjugation is indicated in the following measurements: animals full of food from standard cultures measure $149 \times 58\ \mu$; when practically devoid of food vacuoles, $143 \times 42\ \mu$; conjugants obtained from such cultures measure on the average $107.1 \times 37.7\ \mu$.

We have seen that if the animals in standard cultures are allowed to starve gradually they become reduced in size to that of the conjugants given above, but if this occurs over too long a span of time, conjugation does not follow. The change in size must therefore occur within a given time.

To determine the relationship of previous nutrition to size of conjugants, measurements were made of conjugants obtained from cultures fed and treated in the following ways: (1) control cultures washed free of food 5 days after subculture; (2) similar cultures fed $7^1/_2$ drops of a standard suspension of bacteria per $^1/_2$ cc. concentrate from 5 cc. of the culture; (3) cultures set out 15—16 days after subculture and after washing refed with 5 drops of bacterial suspension per $^1/_2$ cc. concentrate from 10 cc. of culture; (4) cultures fed *Tetrahymena glaucomiformia* (FURGASON, 1937) a small ciliate which can be grown in large numbers in pure culture on 1.0% yeast extract in distilled water.

A word of explanation for (4) is necessary before proceeding with the results. Provided the mouth is of adequate size, *Blepharisma* can engulf and use *Tetrahymena* as food. Thus only if the tetrahymenas have been starved for at least 24 hours after washing and the blepharismas are from well fed cultures and still full of food vacuoles, therefore possessing distended mouth parts, will the blepharismas be able to ingest tetrahymenas. If one adds unstarved tetrahymenas, only a few blepharismas have mouths large enough to engulf tetrahymenas; one obtains a paradoxical result — the blepharismas conjugate in the midst of plentiful food, which is, however, inaccessible because of its size. When tetrahymenas are engulfed, a very large size is attained (203 by 89 μ); when such large animals are starved, they undergo rapid divisions to produce smaller animals which in absence of food may conjugate. Epidemics obtained with progeny of Tetrahymena-fed animals were, however, never as good as those obtained in the other cases, in most cases a maximum at a given time of about 20% in conjugation being observed.

The data in Table 1 summarizing the size studies and measurements indicate clearly that the size of conjugants is not constant but depends upon the previous nutrition, so animals which are very large before the nutritive change is operative will be larger in conjugation. The range in size of conjugants, while variable as indicated, is certainly much smaller than the range in size of the vegetative forms of the various groups. Thus the average size of *Tetrahymena*-fed blepharismas is $203 \times 89\ \mu$, yet the average size of conjugants is $132.5 \times 52.3\ \mu$. Normally the well-fed individual divides to produce a number of smaller individuals before conjugation can be induced, and the reduction in size may be interpreted as regulation towards the more normal species size, as discussed in another paper (GIESE, 1938 b). The bacteria-fed animal full of food vacuoles measures $149 \times 58\ \mu$, when

Table 1.

Size of conjugants as correlated to previous nutrition.

Age and food	Size* in μ of larger conjugant	Size in μ of smaller conjugant	Average size in μ	Probable errors			
				Larger conjugant		Smaller conjugant	
				Length	Width	Length	Width
15-day refed bacterial suspension	$88 \pm 12.5 \times 32 \pm 4.0$	$79 \pm 9.5 \times 29 \pm 5.0$**	91.1×33.4	8.43	2.70	6.40	3.37
	$98 \pm 10.1 \times 32 \pm 3.7$	$87 \pm 7.1 \times 31 \pm 3.9$		6.81	2.50	4.78	2.63
	$106 \pm 10.3 \times 36 \pm 5.4$	$97 \pm 10.3 \times 34 \pm 5.1$		6.85	3.60	6.85	3.44
	$91 \pm 9.5 \times 30 \pm 4.5$	$83 \pm 7.6 \times 27 \pm 4.7$		6.40	3.02	5.12	3.17
5-day control	$111 \pm 9.0 \times 35 \pm 3.7$	$101 \pm 8.3 \times 31 \pm 4.1$	107.1×37.7	6 07	2.50	5.60	2.76
	$112 \pm 7.6 \times 44 \pm 6.8$	$105 \pm 7.9 \times 40 \pm 6.4$		5.13	4.58	5.33	4.32
	$111 \pm 7.1 \times 38 \pm 4.2$	$101 \pm 8.8 \times 37 \pm 3.4$		4.79	2.83	5.94	2.29
	$109 \pm 14.6 \times 39 \pm 6.0$	—		9.44	4.05	—	—
5-day refed	$109 \pm 4.4 \times 43 \pm 4.5$	$104 \pm 9.2 \times 39 \pm 5.5$	111.1×41.8	2.97	3.04	6.20	3.70
	$121 \pm 11.5 \times 44 \pm 4.2$	$110 \pm 11.4 \times 43 \pm 5.7$		7.25	2.84	7.18	3.84
	***$114 \pm 8.1 \times 43 \pm 6.4$	$104 \pm 8.8 \times 41 \pm 4.5$		5.46	4.32	5.94	3.09
	$116 \pm 14.9 \times 40 \pm 7.1$	—		10.04	4.78	—	—
	***$122 \pm 10.1 \times 44 \pm 5.5$	$109 \pm 11.3 \times 38 \pm 17.3$		6.81	3.71	7.12	11.30
Progeny of Tetrahymena fed	$129 \pm 11.6 \times 49 \pm 8.4$	$118 \pm 13.0 \times 45 \pm 7.2$	132.5×52.3	2.82	5.66	8.76	4.86
	$149 \pm 14.3 \times 56 \pm 8.3$	$134 \pm 15.4 \times 54 \pm 8.4$		9.64	5.60	10.40	5.66
	$138 \pm 15.3 \times 57 \pm 10.1$	$127 \pm 15.1 \times 53 \pm 9.7$		10.31	6.81	10.20	6.54

* Each mean the average of 25 measurements followed by the standard deviation.

** 16-day old culture.

*** Fed only 5 drops instead of $7\frac{1}{2}$ as in the others.

practically devoid of food vacuoles, $143 \times 42\ \mu$; the conjugants are always smaller; those refed before conjugation being on the average $111.1 \times 41.8\ \mu$, while those obtained from a 5-day culture in which conjugation was induced without refeeding are on the average $107.1 \times 37.7\ \mu$. The latter difference lacks statistical significance since the error in measurement is of the order of $2.5\ \mu$, but it is suggestive. The other differences are statistically significant. Also animals from a 16-day old culture which are on the average $78.8 \times 23.9\ \mu$ did not conjugate when fed only one drop of bacteria; after feeding with 5 drops they had increased in size considerably before conjugation could be induced, although there is a decrease again before conjugation sets in, and the average size of conjugants was $91.1 \times 33.4\ \mu$. In all these cases the change in size must occur within a certain time lapse before conjugation occurs. Whether the decrement in size and the rate of decrease is comparable for the different nutritional levels of vegetative cells preceding conjugation is not known and awaits development of a good means of determining the volume of the cells as well as greater refinement in the culture methods and control of conditions.

On a few occasions observations were made of three individuals fused in conjugation instead of two. Generally the triple fusions occurred in densely crowded cultures. Such groups are illustrated in Pl. 8 Fig. 7, 8 and 9. While the animals of Pl. 8 Fig. 7 and 8 are comparable in size, one of the animals in Pl. 8 Fig. 9 is much smaller (the larger animals are from *Tetrahymena*-fed cultures, the small one is from a refed 16-day old culture). When smaller animals are mixed with larger ones and the two groups are washed free of food, small organisms tend to mate with small and large with large, but there are many cases of small-large matings; yet the triple fusions are relatively rare, although there is room along the hypostome of a large individual for several small ones. Some factor other than cytostomal surface apparently governs the fusions of individuals in conjugation; the occasional triple fusions are due to some unknown favorable combination of circumstances. Their rarity is suggestive of some sort of regulation. It may be that after two animals have begun to fuse there is a change in the nature of the membrane (suggestive of the fertilization reaction of the metazoan egg) which alters it and makes it impossible, with the rare exceptions noted, for other individuals to attach except loosely and transiently.

In conclusion it may be stated that the experiments carry one step further the correlation of the environmental changes with the onset

of conjugation. A decline in the nutritive state results in a reduction in size. If this occurs within a certain time, conjugation ensues. If the same size is reached over a longer time lapse, conjugation does not occur. The size of conjugants for a given clone under a given set of conditions is not fixed but depends upon the mean size of the population at the time the decline in food occurs.

Summary.

1. Conjugation in a clone of *Blepharisma* was observed to follow a sudden diminution of available food.

2. Additions of bacterial food delayed conjugation, the delay being proportional to food added.

3. A slight feeding of starved animals followed by a sudden decrease of food may result in conjugation, the amount of feeding required depending upon previous starvation.

4. Conjugants obtained by starving animals previously abundantly fed are larger than conjugants obtained by a slight refeeding followed by starvation of animals subjected to prolonged starvation. Conjugants obtained from animals fed a small protozoan are larger than either of the above.

5. The range of size of conjugants is much smaller than the range in size of the population at large, but there appears to be no fixed size to which animals must be starved before conjugation occurs, the size depending upon the previous history.

Bibliography.

Calkins, G. N. (1912): The paedogamous conjugation of Blepharisma undulans Stein. J. Morph. a. Physiol. **23**, 667—688.

Dawson, J. A. (1928): A comparison of the life cycles of certain ciliates. J. of exper. Zool. **51**, 199—208.

Furgason, W. H. (1936): A contribution to the morphology and the taxonomy of some holotrichous ciliates. Thesis. Stanford University.

Giese, A. C. (1935): The rôle of starvation in conjugation of Paramecium. Physiologic. Zool. **8**, 116—125.

— (1938a): Reversible bleaching of Blepharisma. Trans. Amer. micro. Soc. **57**, 77—81.

— (1938b): Cannibalism and gigantism in Blepharisma. Ibid. (in press).

— (1938c): Race and conjugation of Paramecium. Physiologic. Zool. **11**, 326—332.

Giese, A. C. and C. V. Taylor (1935): Paramecia for experimental purposes in controlled mass cultures on a single strain of bacteria. Arch. Protistenkde **84**, 225—231.

Jennings, H. S. (1910): What conditions induce conjugation in Paramecium? J. of exper. Zool. **9**, 279—300.

Jennings, H. S. (1911): Assortive mating, variability and inheritance of size in conjugation of Paramecium. Ibid. **11**, 1—134.

Pearl, R. (1907): A biometrical study of conjugation in Paramecium. Biometrika (Lond.) **5**, 213—297.

Sonneborn, T. M. (1937): Sex, sex inheritance and sex determination in Paramecium aurelia. Proc. nat. Acad. Sci. U.S.A. **23**, 378—385.

Stolte, H. A. (1924): Morphologische und physiologische Untersuchungen an Blepharisma undulans Stein. Arch. Protistenkde **48**, 245—301.

Watters, F. (1912): Size relationships between conjugants and non-conjugants in Blepharisma undulans. Biol. Bull. Mar. biol. Labor. Wood's Hole **23**, 195—200.

Legend to Plate.

Plate 8.

Equivalent initial magnification and enlargement for all figures.

Fig. 1. A bacteria-fed animal from a standard culture. Note the food vacuoles.

Fig. 2. A *Tetrahymena*-fed animal; note numerous food vacuoles with the small protozoans inside.

Fig. 3. A large individual conjugating with a small one. The large individual is the offspring of a cannibal and still contains the remains of a *Blepharisma* in the dark vacuole.

Fig. 4. A pair of conjugants and a non-conjugating animal from a standard culture washed free of food on the fifth day after subculture.

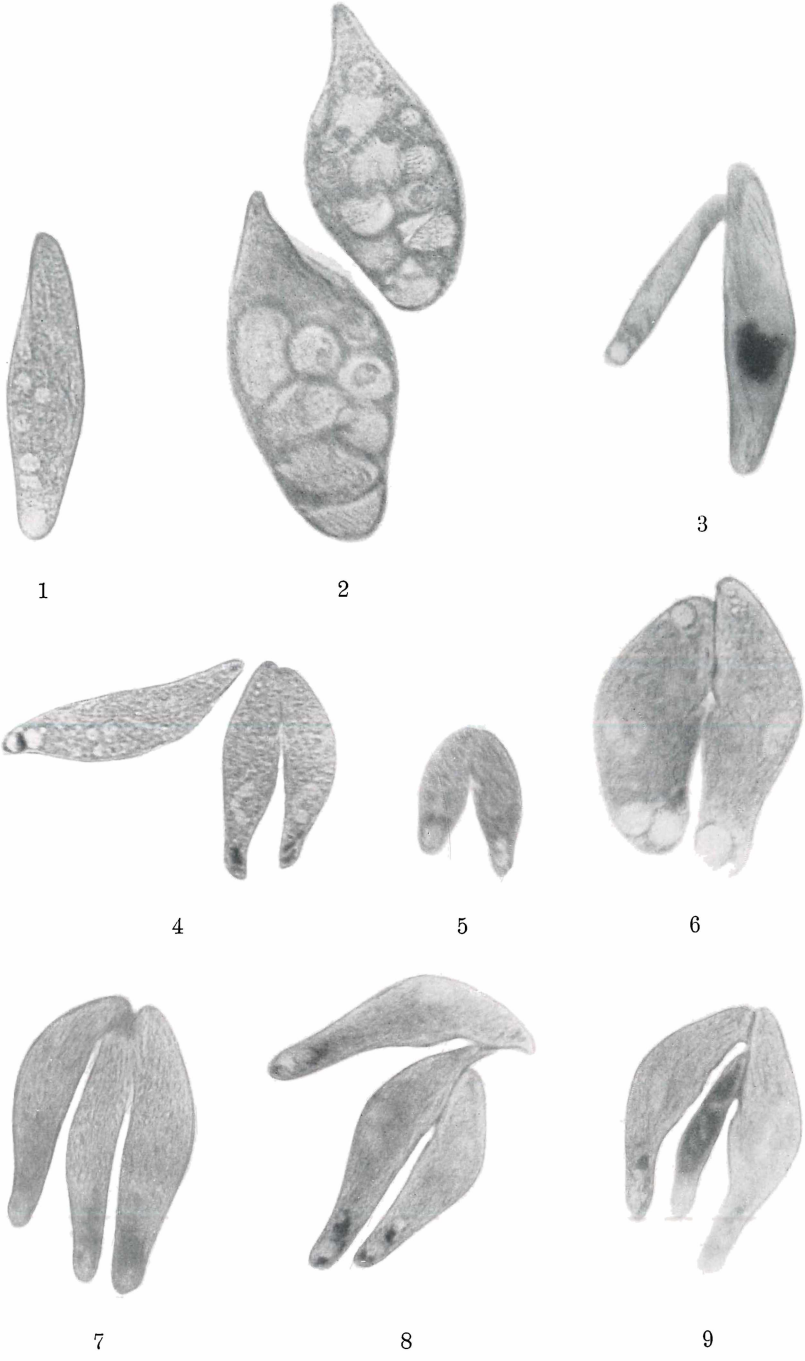
Fig. 5. A pair of small conjugants from a concentrate of animals in a culture 16 days after subculture refed and allowed to starve.

Fig. 6. A pair of large conjugants obtained from a culture fed, before starvation, on *Tetrahymena*.

Fig. 7. A group of 3 animals in conjugation; obtained from a culture previously fed *Tetrahymena*.

Fig. 8. Another group of 3 animals conjugating. Note that attachment is different from that in Fig. 8.

Fig. 9. A group of 3 animals conjugating in which one of the conjugants is much smaller than the others. The small animal is from a 16-day old standard culture.



Giese.

VERLAG VON GUSTAV FISCHER IN JENA