**The Sex-Chromosome of a Stone-Fly,
Acroneuria jezoensis OKAMOTO (Plecoptera)**

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(With 11 text figures and 5 tables)

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Introduction

Since SCHÖNEMUND (1912) reported an ovary-like organ, annexed to the testis, in the male of some species of *Perla*¹⁾, the attention of cytologists has been attracted to this particular example of hermaphroditism along with the BIDDER's organ found in *Bufo*. The physiological function of this organ in question, however, was entirely obscure to him.

A cytological study was first made on this interesting organ by JUNKER (1923), who dealt with *Perla marginata*. He discovered, in this species also, such a doubtful organ, and reported an interesting fact, that the chromosome found in the cells developed in the so-called male ovary shows no difference in features from that of the testis to which the former is appended. The destination of this kind of cells still remains an unsolved question.

At first, the present author commenced a study with the expectation of contributing in some way to the physiological functions of the cells in question, if he could find ovaries of similar kind in the male body of any Japanese species of Perlidae. For this purpose, many different species of Perlidae, inhabiting the waters near Sapporo, were collected. Unfortunately, however, the author was unable to find out a similar occurrence in any of the species studied. The study has necessarily, therefore, not been extended to the male ovaries but has been confined to the cells of the genital organs of normal structures.

As the first report in a series of studies the author wishes to describe in this paper the result obtained from observation on the

1) *Perla maxima*, *P. marginata*, *P. cephalotos*.

chromosomes, both in male and female, especially the sex-chromosome and its behavior during spermatogenesis of *Acroneuria jezoensis*.

Up to this time two studies on spermatogenesis have been reported from Perlidae, one by NAKAHARA (1919) and the other by JUNKER (1923) in connection with the male ovaries, as stated above. From either of them the present result evidently differs not only in the chromosome number but also in the constitution of the sex-chromosome.

The work has been carried on under the direction of Professor K. OGUMA, to whom the writer wishes to express hearty thanks for his continuous encouragement and kind advice. He is also greatly indebted to Dr. H. OKAMOTO in identifying the names of the material.

Material and Technique

Plecoptera spend their larval life in the gravel and stone beds in brooks and rivers. All material used by the writer was obtained from a stream at Garugawa near Sapporo. About 100 male larvae were used in this study, and they were collected from the beginning of April up to about the middle November.

For the fixation, several reagents were applied. But FLEMMING's solution, both strong and reduced in acetic acid, CHAMPY's mixture, and BOUIN-ALLEN's solution (ALLEN, 1918) seemed to give the best results. Smear preparations with acetocarmine were also tried.

In the process of imbedding the material in paraffin, each duration in absolute alcohol, chloroform, and paraffin was shortened as far as possible. Only twenty to forty minutes penetration was applied in melting paraffin. Sections were cut from 2.5 to 5 micra thick. Iron haematoxylin, with or without counter staining of light green, and triple staining method respectively were applied for staining. The safranin-methyl green-acid fuchsin mixture was employed for spindle demonstration.

Observation

1. Development of the Testis

The larva of this species lives in water several years. It is carnivorous and moults several times a year. It emerges out of the water at the middle July after its long aquatic life.

The testis consists of many spherical follicles, about seventy or eighty in number, surrounding a Vas deferens, and presents an acinus-shape in general appearance (Fig. 1). Every follicle is enveloped by the follicle membrane. This membrane contains extremely slender nuclei at intervals, but is pretty thick as compared with the cyst membrane. The nuclei of the cyst cells, composing cyst membrane, are large, and their form varies according to their positions. From September to November of the year previous to its emergence, the testes are yet small, spermatogonia are now furiously undergoing divisions, and spermatocytes in the early growth-period are seen on the inner side of the follicles. The best figures of the spermatogonial divisions are obtained during this period.

In the following March, the testes are almost filled up with spermatocytes in their growth-period, but the meiotic division still does not take place. In this period, there are also very few secondary spermatogonia in late development. The meiotic division commences in the later part of March and continues till the end of July. The spermioteleosis is observed also during July in parallel with the maturing division. For that reason, in the material collected in March or June, the arrangement of the spermatogonium, the first and second spermatocyte and the spermatid group can be distinguished at the same time in a follicle. Whole germinal cells in one cyst, descended from one stem mother cell, are almost in the same division phase; in other words, all cells contained in one cyst are nearly synchronal in development. In the adult, the testicular follicles are entirely filled with spermatozoa.

Fig. 1. Testis of a larva (body length 1.9 cm., testis 0.44 cm.)

2. Period of Multiplication of Spermatogonium

The multiplication period of the spermatogonium can be divided into two different stages, designated as primary and secondary. The primary spermatogonial multiplication takes place in the young larval stage, and the divisions are repeated not rapidly as compared with those of the secondary spermatogonia. JUNKER (1923) reported, in

Perla marginata, that the number of divisions during the entire multiplication period is more than eight. In this species it is just nine, as ascertained by counting the number of primary spermatocytes contained in a cyst. Among them, the first five divisions are of the primary spermatogonium, and the remaining four the secondary.

The primary spermatogonium assumes a large pear-shape having a large nucleus. The cells in one cyst are less than thirty-two in number, and lie in one layer close to the cyst membrane (Fig. 2).

Fig. 2. Primary spermatogonia in 16-cell stage. F.M., follicle membrane; F.M.N., nucleus of the same; C, cyst; c.c., cyst cell.

The secondary spermatogonium is, on the contrary, very small as the result of successive divisions. These seem to be more rapidly repeated than in the case of the primary spermatogonium.

3. Chromosomes in the Spermatogonial Metaphase

A spermatogonium possesses twenty-five chromosomes as a diploid number, as is quite easily counted in the mitosis of the nucleus (Fig. 3, *a, b, c, d*). Of these, five chromosomes are bent rod-shaped of exceedingly large size. They take an outer position in metaphase arrangement and are most clearly distinguishable from the remainder. The remaining twenty are all straight rod-shaped constituting ten homologous pairs, of which six are of medium size and four are markedly small.

Special attention should be paid here to the five chromosomes of large size. Two of them are U-shaped with nearly equal arms, while another two are J-shaped. These are considered as two homologous pairs of autosomes. The remaining one, however, is V-shaped with arms strongly opened, and accompanies no homologous partner at all. This chromosome is evidently considered as the sex-chromosomes, as ascertained not only by the observation of the chromosomal behavior throughout the process of the spermatogenesis, but also by comparison with the chromosomes of the female.

Now let us examine the nature of the sex-chromosomes in detail. As shown in Fig. 3, the J-shaped chromosome differs very much in form from the others. But the U-shaped ones have much resemblance to the sex-chromosomes. Cases were found where the latter is hardly discriminated from

the former in so far as the form is concerned. First of all, however, the sex-chromosome always has a tendency to stretch or open out its arms, instead of inward curving as those of the U-shaped autosomes (Fig. 3, *a*, *b*, *c*).

The second point, by which the sex-chromosome differs from the U's, is the relative length of the two arms, which are unexceptionally unequal, while they are nearly equal in the U's. The more detailed investigation on the length of the sex-chromosome will be described in a later section. In connection with the difference of arm length, one more important feature is to be discovered in the sex-chromosome; that is two conspicuous constrictions, one of which is found at a point near the apex of the V, to which the spindle fibres are attached, and the other at a sub-terminal point of the short arm (Fig. 4).

Fig. 4. X-chromosome having two distinct constrictions.

Fig. 3. Spermatogonial metaphase plates. $\times 1800$. X, X-chromosome; A, large U-shaped chromosome; B, large J-shaped chromosome.

These two constrictions appear in the sex-chromosome not only

during the growth-period but also in the maturing division of the primary spermatocyte. In contrast to such clearness, however, it is scarcely possible to discover them in the sex-chromosome which accomplished the final condensation and acquired strongest capacity of staining.

4. Chromosomes in the Growth-Period and Prophase of the First Spermatocyte Division

Generally speaking, the internal changes during the course of the growth-period of the primary spermatocyte are comparatively simple in this species. The stages of prochromosomes, leptotene, synizesis or bouquet, diplotene and second contraction are abbreviated. The homologous chromosomes in the final gonial telophase complete their pairing, which seems to have begun in the multiplication period, about the time when they begin to disintegrate. This is an important point characteristic to this species. The diffusion stage directly follows (Fig. 5, *b*). Throughout this stage the chromatin spiremes lose their basophily in considerable degree, still preserving their individuality (Fig. 5, *c*). Then the spiremes grow gradually longer and subsequently narrower, and recover their basophilic nature (Fig. 5, *d*). While the nucleus continues its growth the pachytene threads involved therein again lose their basophily, and give rise to a nucleus of similar appearance to

Fig. 5. Nuclei of the primary spermatocytes in the growing period. $\times 1600$. *a*, disintegration stage; *b*, diffusion stage; *c*, the same; *d*, pachytene stage; *e*, cryptosome stage; *f*, later cryptosome stage; *g*, early diakinesis; *h*, diakinesis.

that of the diffusion stage, but remarkably larger (Fig. 5, *e, f*). This stage evidently corresponds to the cryptosome (McCLUNG) or confused stage (WILSON). After a short duration this stage advances to the diakinesis, in which, the chromosome threads begin to condense so as to be converted into the tetrad (Fig. 5, *g, h*). The nucleus attains its maximum dimension during the diakinesis.

During the successive stages above described, there is found without exception, a chromatin nucleolus, which represents the sex-chromosome. In the final gonial disintegration stage it usually takes the shape of a horse-shoe as seen in Fig. 5, *a*, but changes its form later in various

Fig. 6. Various forms of X-chromosome in cryptosome stage.

ways. Briefly, the sex-chromosome grows gradually narrow, by increasing its length (Figs. 6 and 7), and very often bends like a V or U. Constrictions, median and sub-terminal, are frequently observed, especially when the chromosome does not bend strongly, but extends

Fig. 7. Various forms of X-chromosome in early diakinesis.

two arms rather horizontally (Fig. 6, *c* and Fig. 7, *f*). When the diakinesis sets in, the sex-chromosome assumes its final form as a narrow V, bent

in an acute angle at the median constriction (Fig. 5, *h*), and it enters the metaphase plate in this form as seen elsewhere in the later stages.

5. Chromosomes in the Metaphase of the First Division

In the heterotypic nuclear plate of the primary spermatocyte, there are counted thirteen chromosomes as the haploid number. Fig. 8, *a* represents all the chromosomes of one plate arranged in serial order. The univalent sex-chromosome is represented by the fifth one from the left in the figure, showing the characteristic V-shape,

and the point of the spindle attachment coincides with that of the spermatogonial metaphase, viz., at the apex of the V. The remaining twelve are represented by bipartite bodies, of which two (A and B in the figure) are exceedingly large and easily distinguishable from the other chromosomes.

In the typical first meiotic metaphase of the present species, the chromosomes are arranged forming two concentric rings upon the equator. Fig. 8, *b* represents the polar view of the equatorial plate in the present division. In the figure, thirteen chromosomes are clearly observed and the two large chromosomes A and B are always found in the outer ring with seven smaller ones. The chromosomes which constitute the inner ring are four in a majority of cases. Contrary to the usual cases, the position of the sex-chromosome in the plate is absolutely confined to the inner ring and it is arranged in such a way that its long axis lies parallel to the spindle axis. Therefore, under a certain focus in the polar view, the sex-chromosome presents a single rod-shape or rather dot-shape like appearance, but,

Fig. 8. Chromosomes in the first maturing division. $\times 1800$. *a*, a complete garniture of metaphase chromosomes; *b*, the same in polar view; *c-f*, the side views of the spindle; *g*, a diagrammatic figure, showing the relation between X-chromosome and the plane of division (*d.p.*); *h-i*, examples in which the constrictions are markedly distinct in X-chromosomes; *j-m*, the side views of the anaphase chromosomes.

tracing its entire form by focussing, one can easily find the two arms, from which it is constituted.

Next, observing the equatorial plate from the side of spindle we can soon find out again that the sex-chromosome occupies the central position of the spindle and stands vertically upon the metaphase plate (Fig. 8, *c, d, e, f*). It seems like a unique example in the equatorial arrangement of the sex-chromosome, because in most of the known cases, where a single X-chromosome is found, it lies, almost without exception, horizontally upon the plate. Such a vertical arrangement of the sex-chromosome, relative to the equator of the spindle, is very frequently found, on the contrary, when it is composed from two different elements, X and Y, as in the cases of mantis (OGUMA, 1921) or *Oecanthus* (MAKINO, 1932). Moreover, our attention is particularly attracted to the phenomenon that the very point of the spindle attachment rests upon the equator as Fig. 8, *g* shows. The two constrictions, which appear in the sex-chromosome during the growing period, often become observable again, and especially the subterminal constriction of the shorter arm is very frequently noticed with perfect clearness (Fig. 8, *h, i*).

When the twelve bivalent chromosomes are divided into their daughter halves in the course of division, the sex-chromosome fails to divide itself and goes to the one pole, towards which the vertex of the V was originally directed. No precession takes place in the sex-chromosome in the course of its migration to the pole, it keeping nearly the same velocity as the divided autosomes (Fig. 8, *j, k, l, m*).

6. Chromosomes during Interkinesis and in the Secondary Spermatocyte

As the cytokinesis is completed in this heterotypic division, the karyoplasmic transparent matter becomes visible in the area surrounding the chromosomes which crowded at the pole. Then the nuclear membrane is first formed along the periphery of this matter (Fig. 9, *a*), and the autosome commences to disintegrate to some extent (Fig. 9, *b*). This defines the interkinesis, although it does not last long, and soon the prophase of the second division follows. In this respect the present species differs from *Perla imaginata*, reported by NAKAHARA (1919), in which no interkinesis occurs.

As regards the sex-chromosome there are two different classes of the second spermatocytes as expected from the mode of division

of the chromosomes. One class possesses thirteen chromosomes, twelve autosomes and one sex-chromosome, and the other merely twelve autosomes without the X. The former is the X-class (Fig. 9, *d*) and the latter is the no X-class (Fig. 9, *e*). The chromosomes

Fig. 9. Interkinesis and the second maturing division. $\times 1285$. *a*, two sister cells (secondary spermatocytes); *b*, interkinesis; *c*, early prophase of the second maturing division; *d-g*, metaphase chromosomes in polar view; *h*, anaphase; *i-j*, spermatids of no X-class (*i*) and of X-class (*j*).

closely resemble those found in the spermatogonium in general aspect as well as in arrangement, and the sex-chromosome lies horizontally upon the equatorial plate in contrast to the case of the first division. The subterminal constriction of the sex-chromosome appears very often too in this stage (Fig. 9, *f, g*). All chromosomes are divided along the longitudinal split, and migrate to each pole. The sex-chromosome is also divided, like the autosomes, into two equal halves indicating no particular behavior (Fig. 9, *h*).

The resulting daughter cells, spermatids, acquire first the nuclear membrane and the autosomes begin to disintegrate into fine chromatic granules scattered evenly through the nucleus (Fig. 9, *i*). But the sex-chromosome remains in compact form for some time; thereby its identification is quite easy in this kind of cells (Fig. 9, *j*).

7. Chromosomes in the Somatic Cells of Female

As far as the sex-chromosome is concerned, at least, it is self-evident that the examination of the chromosomes of the female cells is by all means very important in relation to sex-determination. From this point of view the writer examined the cells taken from

the female individuals, taking the somatic cells as the material, on account of the facility with which the work can be carried on.

Excellent figures of female diploid chromosomes in division were found in several numbers in the cells of follicle epithelia and of oviduct, and they are available for the purpose of counting the complete number of chromosomes contained.

Both in the follicle cells (Fig. 10, *a, b*) and in those of oviduct (Fig. 10, *c*), the chromosomes are distinctly counted to the number of twenty-six, one more than the diploid number in the male germ-cells.

Fig. 10. Chromosomes in the female cells. $\times 1800$. *a-b*, cells of follicle epithelia; *c*, a cell of oviduct.

All the chromosomes are short and rod-shaped, excepting six, which are enormous in length in comparison with the remainder. Of these six large chromosomes two are U-shaped and two J-shaped, evidently corresponding to those assuming similar shapes in the spermatogonial metaphase, and the remaining two V-shaped chromosomes (shown black in the figures) represent without doubt the sex-chromosomes in a paired condition. These two sex-chromosomes, in some cases, stretch out their arms nearly in a straight line. (Fig. 10, *b, c*).

From the results obtained from the present observation, the sexual difference of the chromosomes is quite obvious. The difference is due to the number of the sex-chromosomes: both the male and female cells alike constantly contain twenty-four autosomes, but the sex-chromosomes are two in the female, while only one in the male cell as already stated. In this respect the present species belongs to the XO-XX or *Protenor*-type of WILSON, the most common type found in the animal kingdom.

8. Comparison with Other Species

According to NAKAHARA (1919), ten chromosomes appear in the spermatogonial division of *Perla immarginata*. The chromosome group consists of two pairs of V's, a pair of rods, two spherules

(m-chromosomes), and a pair of unequal rods, one being strikingly longer than the other. It is these last ones that he interpreted as X- and Y-chromosomes, respectively. In the polar view of the equatorial plate of the first maturing division, five chromosomes can be seen distinctly. X and Y are joined to each other and appear as a single body. The remaining four are autosomal tetrads including m-chromosome. X and Y separate from each other in this division, thus giving rise to two types of the second spermatocyte according to the sex-chromosomes. The chromosome number in the second spermatocyte is therefore five, consisting of two V's, one rod, 'm', and X- or Y-chromosome. All five chromosomes divide equationally in the second maturing division. The female chromosome group has not been observed.

JUNKER (1923) reported that the diploid chromosome number in the spermatogonium of *Perla marginata* is 22, or 20 homologous and two unequivalent sized heterochromosomes. In the first maturing plate, twelve elements have been seen, ten autosome tetrads and two separate heterochromosomes. As a matter of importance, these two heterochromosomes migrate together towards the same pole in the present division, and the two types of the second spermatocyte are produced. One of them has twelve chromosomes and the other has ten chromosomes. The diploid chromosome number in the oogonium of the female individual is 24. All are paired chromosomes including 4 heterochromosomes. The oogonium found in the so-called ovary of male possesses twenty two chromosomes, similar to the spermatogonium, and therefore differs from the oogonium just mentioned. From what JUNKER (1923) observed, we know the sex-chromosome of the species is XO-type in which the X is represented by two elements of unequal size. This might have resulted if chromosome fragmentation would have taken place in the single X, which is expectable when we recall the occurrence of constrictions as observed in the present study. But, the size of the X-chromosome exhibits such a remarkable difference between these two species, that one can hardly take them as of the same category. Thus all three species studied up this time present least accordance with each other in respect to the morphology of the X-chromosome.

9. Length of the Sex-Chromosome

In this section the writer wishes to mention some statistical investigations on the sex-chromosome. The writer first examined the

Table 1
Measurement of two arms of the X-chromosome, under magnification of 2500 times. *l*, long arm; *s*, short arm;
l+*s*, total length; *l*−*s*, difference in the two arms; *l*/*s*, ratio of two arms.

Indi- vidual	Cyst	Number ob- served	Length of long arm			Length of short arm			<i>l</i> + <i>s</i>			<i>l</i> − <i>s</i>			<i>l</i> / <i>s</i>
			max.	min.	aver- age	max.	min.	aver- age	max.	min.	aver- age	max.	min.	aver- age	
A	I	14	1.45	1.10	1.260	1.17	0.85	1.028	2.62	1.92	2.297	0.36	0.05	0.21	1.21
B	II	10	1.15	0.85	0.989	1.05	0.75	0.822	2.11	1.60	1.811	0.27	0.07	0.17	1.20
C	III	19	1.12	0.87	1.00	0.90	0.62	0.763	1.96	1.49	1.714	0.41	0.11	0.22	1.30
	IV	7	1.04	0.85	0.960	0.81	0.60	0.707	1.85	1.53	1.665	0.33	0.19	0.25	1.36
D	V	18	1.10	0.85	0.951	0.93	0.70	0.748	2.02	1.55	0.735	0.40	0.05	0.17	1.21
	VI	15	1.08	0.82	0.942	0.88	0.64	0.782	1.92	1.46	1.718	0.30	0.04	0.17	1.20
	VII	16	1.04	0.77	0.920	0.86	0.69	0.763	1.82	1.46	1.681	0.28	0.04	0.17	1.20
	VIII	12	1.17	0.78	0.930	1.08	0.68	0.812	1.87	1.46	1.939	0.18	0.06	0.11	1.14
Total	8	111	1.45	0.77	0.993	1.17	0.60	0.811	2.62	1.46	1.795	0.41	0.04	0.181	

quantitative relation between the two arms of the sex-chromosome which differ in length, and next the variation in length occurring in both arms and subsequently in the sex-chromosome. At all events, it undoubtedly consists of two or three elements, though it could not be known what significance the constrictions have, in the range of the present study.

For this purpose 8 cysts from 4 different individuals, which are similar in the fixing method or the collecting time, were observed: one cyst in two individuals, two cysts in one individual, and four cysts in the other individuals. And the observed cells are 111 in total. As all cells which are contained in the same cyst are originated from one primordial germ cell, all cells examined are grouped in 8 groups according to the cysts. All are calculated in the equatorial plate of the first maturing division. The measurements were undertaken with a ZEISS 2 mm. objective and a compensation ocular 20, with a tube length of 160 mm. at the desk level.

From this table 1 it becomes clear that the length of long- and short-arms, the total length, and the difference in length of the two arms differ in each individual. One individual A has strikingly larger sex-chromosomes, than the others. In the individual A, maximum and average value of the long arm are 1.45 and 1.260 respectively, and of the short arms 1.17 and 1.028 respectively, while in other individuals the maximum and average value of the long arms are nearly 1.10 and 0.95 and of the short arms nearly 0.90 and 0.80 respectively. Accordingly the total length of the sex-chromosome of the individual A is also much larger than the others, i.e., the maximum and average value of the former are 2.26 and 2.297 respectively, while nearly 2.0 maximum and 1.7 average value are found in every other individual. On the other hand, the ratio in length of the two arms of the individual A, 1.21, is almost equal to those of the others except in individual C in which the value of the ratio is represented by 1.3. The above mentioned fact indicates that the length of the sex-chromosome and the ratio in length of the two arms are different in each individual. While, on the contrary, examining all cells found in every cyst of an individual, not only the value of maximum, minimum and average length of the two arms, the total length, the difference in length, but also the ratio of the two arms are nearly always almost equal. I could not examine whether the individual A, which has the large sex-chromosomes, indicates some special morphological differences from the others. And in total of

all cells measured, the maximum, minimum and average value of the sex-chromosome are as follows:

Table 2

	Maximum	Minimum	Average
Long arm	1.45	0.77	0.993
Short arm	1.17	0.60	0.811
Total length	2.26	1.46	1.795
Difference	0.41	0.04	0.181

Next, the variation in length of the sex-chromosome, the long, short arm and the total length, are tabulated in Tables 3, 4, and 5

Table 3

Frequency of the length of the long arm.

Class	0.80	0.85	0.90	0.95	1.00	1.05	1.10	1.15	1.20	total
Frequency	3	11	15	22	21	14	7	2	2	97

Table 4

Frequency of the length of the short arm.

Class	0.65	0.70	0.75	0.80	0.85	0.90	0.95	1.00	1.05	1.10	total
Frequency	5	15	21	26	16	9	2	0	1	2	97

Table 5

Frequency of the total length.

Class	1.55	1.65	1.75	1.85	1.95	2.05	2.15	2.25	total
Frequency	7	22	28	22	12	4	1	1	97

respectively. As shown in these tables, the variation is clearly a fluctuating variation in all cases. But individual A is excluded in these calculations, as its variation is too large. In the long arm (Table 3) we find the greatest frequency 22 in the 0.95 class, while in the short arms (Table 4) the 0.80 class acquires the highest value of frequency. In total length of the sex-chromosome (Table 5) the class 1.75 has the highest value in number, 28 cases in 97.

10. Chromatoid Body

Only one small spherical chromatoid body is found during both spermatocyte-divisions in the present species. It is deeply stained by haematoxylin just as chromosomes are stained, but never by safranin. And even in the haematoxylin staining it can clearly be distinguished from the chromosomes without difficulty, whenever we carefully observe the plate, because it never enters the spindle as does the

Fig. 11. Chromatoid bodies. $\times 1800$. *a-b*, primary spermatocytes; *c*, secondary spermatocyte; *d-e*, spermatids; *ch*; chromatoid body.

chromosome, but always remains in the surrounding cytoplasm. It fails to divide in either mitosis, passing over bodily, first into one of the daughter spermatocytes and then into one of the spermatids (Fig. 11). The chromatoid body, therefore, enters only one spermatid out of four, similar to the case of the hemipter *Pentatoma senilis* (WILSON, 1913).

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