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THE DRUG-FASTNESS OF SPIROCHETES TO ARSENIC, MERCURIAL, AND IODIDE COMPOUNDS IN VITRO.

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It has been known for some time that in trypanosomiasis the trypanosomes which have survived the first effect of an arsenic germicide, such as atoxyl or arsacetin, offer a greater resistance to a subsequent dose of the same drug. By subjecting the organisms to repeated injections of an arsenic medicament, one tends to create, both in animals and in man, a strain or race which resists the arsenic chemotherapy to such an extent that the term arsenic-fastness has come into existence as a brief designation of the modified strain.[1] A similar phenomenon has been observed in spirochetosis in fowls and mammals. Even with a typical bacterium, there seems to exist a possibility of raising the original resistance against a certain arsenic compound to a considerable degree, as was shown by Marks[2] in his experiment with the paratyphoid bacillus, which he was finally able to cultivate in a medium containing eight times the amount of arsenious acid which the organism was able to withstand at the beginning of his experiment. Marks accomplished this within a period of 3 years by successive transplantations from a weaker to a gradually stronger concentration of the acid.

In syphilis, even before the discovery of *Treponema pallidum*, it had long been suspected that the causative agent of this disease acquires a gradual tolerance to the action of mercurial and iodide preparations. The intermittent form of treatment generally adopted by clinicians, with gradually ascending doses of the medicaments for each course, bears sufficient evidence of this assumption. That

[1] Ehrlich, P., and Hata, S., Die experimentelle Chemotherapie der Spirillosen, Berlin, 1910.

[2] Marks, L. H., Ueber einen arsenfesten Bakterienstamm, *Z. Immunitätsforsch., Orig.*, 1910, vi, 293.

unicellular organisms, under certain environmental influences, gradually acquire tolerance to certain toxic substances is not inconceivable, inasmuch as instances are not wanting where a group of cells, or an entire organization of much higher multicellular organisms, becomes adjusted to new surroundings when the change is brought about by degrees. In short, the state of drug-fastness is well known among the metazoan organisms.

To establish definitely whether *Treponema pallidum* acquires an increased resistance to various antisyphilitic therapeutic agents is of great practical importance, for the regulation of dosage of medicaments must necessarily be guided by the changes which occur at the same time in the resistance of the parasites. It may be recalled that Ehrlich assumed from analogy with trypanosomiasis and fowl spirochetosis that *Treponema pallidum* becomes readily fast or insensitive to the action of arsenic compounds, and with this idea he evolved his *therapia sterilisans magna*, that is, a total sterilization of *Treponema pallidum* in the system of the infected individual by a single administration of salvarsan, in which he combined a maximum parasitotropic and a minimum organotropic activity. The benefit derived from his efforts to achieve the aim of *therapia sterilisans magna* has been great, but in its practical application salvarsan has fulfilled only a part of Ehrlich's expectation. The consensus of opinion is that salvarsan has to be used in many successive doses, like other antisyphilitic medicaments, in order to obtain the full benefit of the drug. The introduction of salvarsan and neosalvarsan as routine medicaments in the treatment of syphilis raises once more the question whether or not the repeated injections of these preparations tend to produce an arsenic-fast strain of *Treponema pallidum*. This vital point, as well as those with regard to the alleged fastness of the *pallidum* against mercurial and iodide compounds, has never been proved experimentally, probably because of the fastidious character of *Treponema pallidum* in its behavior in the experimental animals available for studying these points.

For ascertaining experimentally that the syphilitic organism becomes gradually more tolerant to the sterilizing effect of various arsenic, mercurial, and iodide preparations, four different methods suggest themselves: (1) to determine *in vivo* the resistance of *Trep-*

onema pallidum to the germicidal action of the drug in question, both before and after the administration of the drug; (2) to subject *in vitro* the *pallidum* derived from normal and treated animals to the action of various concentrations of the drug and then to inoculate them into a series of suitable animals; (3) to cultivate the organism instead of inoculating it into animals as in (2); (4) to determine the degrees of tolerance of pure cultures of *Treponema pallidum* to a given medicament by testing the organisms *in vitro* against gradually increasing doses of the drug.

The first two methods are practically unavailable for the present problem, since there is no animal in which an accurate estimation of the relation between the number of the parasites and the severity and character of the lesions produced could be made with uniformity, and no method is yet known which will permit the cultivation of the *pallidum* directly from the animal tissue emulsions, as would be required if we were to adopt the third procedure. We have, therefore, left these three methods out of consideration and followed the fourth, the only procedure which may be carried out with the various factors under ready control and with a fair degree of constancy in results.

In the present work, we have chosen not only the syphilitic spirochete, but also some allied non-pathogenic varieties, and tested them against some of the representatives of antisyphilitic medicaments. We do not consider that the phenomena observed *in vitro* on pure cultures of spirochetes can be directly transferred to the processes occurring in the animal or human body, but we believe that the observations reported in this paper warrant sufficient interest as such.[3]

EXPERIMENTAL.

Method.

As the test objects, three strains of pure cultures of *Treponema pallidum*, one of *Treponema microdentium*, and one of *Spirochæta refringens* were employed. Against each of the above organisms, salvarsan, neosalvarsan, bichloride of mercury, and Lugol's solution

[3] Akatsu, S., The Resistance of Spirochetes to the Action of Hexamethylenetetramine Derivatives and Mercurial and Arsenic Compounds, *J. Exp. Med.*, 1917, xxv, 363.

were used. The solutions of salvarsan and neosalvarsan in sterile distilled water were each time freshly prepared, the proportion being 1 : 10,000. The solution of bichloride of mercury was made up to 1 : 10,000 during the early part of the experiment and became 1 : 1,000 as the concentration was gradually increased in order to keep the volume of the solution of the sublimate to be added to the media within a certain limit. Lugol's solution was prepared by dissolving 1 gm. of iodine and 2 gm. of potassium iodide in 300 cc. of distilled water. In the experiment a 1 : 10 dilution was first used, then an undiluted solution, (1 part of iodine, 2 parts of potassium iodide, and 300 parts of distilled water), and finally one three times as concentrated as the usual undiluted solution (1 part of iodine, 2 parts of potassium iodide, and 100 parts of distilled water).

Because of certain technical difficulties, the use of solid media was restricted to a small series. The majority of the experiments were carried out by means of a fluid medium consisting of equal parts of neutral bouillon and ascitic fluid with a piece of fresh kidney of a normal rabbit. To maintain the relative volumes of the drug solutions and the culture media, none of the former was added in a volume greater than 1 cc. or less than 0.05 cc. When the requisite amount of the drug solution exceeded 1 cc., a corresponding quantity of a tenfold stronger concentration was employed in order to keep the volume within the specified limit. The total volume of the culture medium in each tube was made uniformly 5 cc., including the quantity of drug solution added.

A series of test-tubes was set up, each containing a piece of fresh rabbit kidney, and inoculated with the pure culture of the spirochete. To the tubes were added first the ascitic bouillon (equal parts), and then the drug solution in the quantity indicated for each tube. The whole content of the tube was thoroughly mixed by gently inverting the tube a few times, then covered with a layer of sterile paraffin oil, and finally incubated at 36°C. in an anaerobic apparatus. At the end of 14 days, the cultures were examined for growth under the dark-field microscope. From the tubes showing varying degrees of growth, transfers were made into a new series of media containing different doses of the drug. Again, after a fortnight's incubation, transfers were similarly made, the process being repeated every 2 weeks.

Results of Experiments.

It was possible to find certain quantities of each drug, the addition of which to the culture media completely inhibited the growth of the organisms. As the quantities of the drug were gradually reduced, the organisms grew in corresponding abundance. With a very small quantity of the drug, no appreciable effect upon the growth was seen. But between the two extremes there were doses which distinctly showed the inhibitory influence of the drug as indicated by the retardation and meagerness of growth. In the border-line tubes the organisms were fewer in number, less active, and often granular in appearance. Numerous degenerated forms were seen in these tubes.

To conform with the purpose of the present work, transfers were made both from the border-line tubes and from those containing smaller amounts of the drug into a number of new media, to some of which ascending doses of the drug solution had been added. After 14 days in the incubator at 36°C., the cultures were examined for growth in the manner already described. From repeated observations it was discovered that no growth or very scant growth was obtainable when a transfer was made from one of the border-line tubes to a tube containing an equal quantity of the drug, and that even if there was any growth in the second transfer, it was not further transferable. The organisms seemed to succumb to the continued effect of the drug in such a concentration. Under these conditions there was no evidence that the organisms had in any way acquired an increased resistance to the drug.

On the other hand, the organisms growing fairly well in the tubes with somewhat smaller amounts of the drug were found capable of transplantation into new media, in the presence of not only the same amount, but also of somewhat larger doses. In other words, the minimal inhibitory doses became gradually larger with each transfer. In the accompanying tables we present a general aspect of the effect of drug-containing media upon the gradual development of tolerance by the spirochetes.

The point of interest here lies in the fact that the organisms under the influence of moderate quantities of an adverse drug acquire a greater resistance to it and are capable of doing so progressively up to a certain point. As will be described, the various organisms em-

ployed in this experiment behaved differently towards different agents, and our results permit the tentative generalization that the group of treponemata gains, under certain conditions, a definite, though rather slight degree of resistance to salvarsan and neosalvarsan, while its resistance to the bichloride of mercury (and iodide?) preparation is strikingly augmented under similar circumstances.

In Tables I and II the reaction of different spirochetes towards the influence of each of the chemical preparations used in the present work is shown.

TABLE I.

First transplantation from drug media to drug media.		Transferred to the tubes containing.				
		Dose.				No drug.
		1	2	3	4	
From tubes containing the doses and showing the growth indicated.	Dose 1, causing total inhibition (−).	−	−	−	−	−
	Dose 2, causing slight growth (< <+).	−	−	−	−	+
	Dose 3, causing moderate growth (<+).	−	<+	+	+	+
	Dose 4, causing good growth (+).	−	+	+	+	++
	Dose 5, causing no inhibition (++).	−	<+	<+	+	++
	Control tube without any drug, showing luxuriant growth (++).	−	< <+	<+	+	++

TABLE II.

Third transplantation from drug media to drug media.		Transferred to the tubes containing.				
		Dose.				No drug.
		1	2	3	4	
From the second transplants containing the doses and showing the growth indicated.	Dose 2, showing moderate growth (<+).	<+	+	++	++	++
	Dose 3, showing good growth (+).	<+	+	++	++	++
	Dose 4, showing good growth (+).	<+	+	++	+	++
	Control tube without any drug, showing luxuriant growth (++).	−	< <+	<+	+	++

Table III represents the results of experiments with salvarsan and shows how various *pallidum* strains, and *microdentium* and *refringens*, withstood the action of the drug.

TABLE III.

Salvarsan.

Maximum Doses in Which Abundant Growth Still Occurred on Successive Transplantations.

	Generation in drug media.						
	1	2	3	4	5	6	7
T. pallidum.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Strain McD.....	0.03	0.05	0.06	0.07	0.07	0.08	0.1
" R.....	0.02	0.03	0.04	0.04	0.05	0.1	0.12
" Z. A.....	0.02	0.04	0.04	0.05	0.08	0.12	Accident
T. microdentium.....	0.01	0.02	0.04	0.06	0.07	0.07	"
S. refringens.....	0.02	0.02	0.04	Accident.	0.04	0.04	0.05

The figures denote the amount in milligrams of salvarsan contained in 5 cc. of the culture medium. The first column shows the doses of salvarsan in which various strains of the spirochetes still thrived; the second those in which the organisms grew well on the first transplantation; the third those for the second transplantation, etc. It will be seen that the initial tolerance of the *pallidum* and *refringens* was slightly greater than that of the *microdentium*, while the rate with which tolerance increased, up to the end of the fifth and sixth transplantation, was greatest with the *microdentium* and *pallidum*, these having attained about six to seven times their original tolerance within $3\frac{1}{2}$ months. The *refringens* still remained sensitive to the drug during this time.

Table IV shows the results with neosalvarsan, and they agree in general with those obtained with salvarsan, except that larger quantities were tolerated on account of its weaker action. The *pallidum* and *microdentium* reached four to five times their initial tolerance, while the tolerance of *refringens* was least affected.

In general, the tolerance of the *pallidum* and *microdentium* to salvarsan and neosalvarsan was definitely raised, while that of the *refringens* seems to have been least increased. With the *pallidum*

TABLE IV.

Neosalvarsan.

Maximum Doses in Which Abundant Growth Still Occurred on Successive Transplantations.

	Generation in drug media.						
	1	2	3	4	5	6	7
T. pallidum.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Strain McD	0.03	0.07	0.08	0.1	0.12	0.15	0.15
" R	0.02	0.05	0.06	0.06	0.07	0.08	0.08
" Z. A	0.03	0.04	0.06	0.06	0.07	0.08	0.1
T. microdentium	0.02	?	0.03	0.07	0.1	0.1	0.1
S. refringens	0.03	0.06	0.08	0.08	0.1	0.1	0.1

strains, the rise in tolerance was gradual and the probable limit was not reached until the fifth or sixth transfer, which was made at the end of the 12th or 14th week. With the *microdentium* and *refringens*, especially the latter, the limit of tolerance was reached much sooner, and it could not be raised further after the fourth transfer. In salvarsanized media, the *refringens* reached the limit on the second transfer and remained unchanged for the rest of the experimental period.

The results obtained with bichloride of mercury, compared with those with salvarsan and neosalvarsan were striking. Table V shows that the initial tolerance of all the spirochetes was almost the same, being 0.01 mg. per 5 cc. of the medium. One of the *pallidum* strains tolerated 0.02 mg. The increase in tolerance rapidly rose from the first transfer and progressed gradually for the following generations. In *Treponema pallidum* the limit was not reached until the 16th week (seventh transfer), while that of the *microdentium* had already been reached on the 8th week (third transfer), and that of the *refringens* on the 12th week (fifth transfer). Even the *pallidum* strains could not tolerate much higher concentrations, the absolute limit being 1 mg. in 5 cc. of the medium, when the color of the tissue, as well as of the ascitic fluid, becomes gray within a few days at 36° C., and black streaks appear in the tissue.

The striking feature of the experiment is the high rate of increase in tolerance through the organisms' becoming accustomed to the

TABLE V.

Bichloride of Mercury.

Maximum Doses in Which Abundant Growth Still Occurred on Successive Transplantations.

	Generation in drug media.							
	1	2	3	4	5	6	7	8
T. pallidum.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Strain McD.	0.02	0.07	0.13	0.17	0.2	0.23	0.5	0.7
" R.	0.01	0.03	0.07	0.1	0.2	0.3	0.7	
" Z. A.	0.01	0.02	0.07	0.07	0.15	0.3	0.5	0.5
T. microdentium.	0.01	0.02	0.07	0.1	0.1	0.1	0.1	0.1
S. refringens.	0.01	0.015	0.03	0.07	0.1	0.3	0.3	0.3

mercurial salt. With the *pallidum*, it was between 35 and 70 times the initial tolerance; with the *refringens*, about 30 times; and with the *microdentium*, 10 times. We did not believe that these delicate organisms could be made resistant to the action of so simple and powerful an inorganic disinfectant as bichloride of mercury. The above observations seem to point to the possibility that bichloride of mercury forms, with certain constituents (proteins and lipoids) of the culture medium, a compound which induces tolerance on the part of the spirochetes.

In Table VI the results obtained with Lugol's solution are given. The figures in the first and second columns do not indicate the maximum doses in which the spirochetes could grow, but those which

TABLE VI.

Lugol's Solution.

Amounts Contained in Successive Transplantations.

	Generation in drug media.					
	1	2	3	4	5	6
T. pallidum.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
Strain McD.	0.03	0.05	0.5	1.5	2.5	2.5
" R.	0.03	0.04	0.5	0.7	1.5	2.5
" Z. A.	0.02	0.05	0.5	1.0	2.0	2.0
T. microdentium.	0.01	0.06	0.3	0.8	0.8	2.0
S. refringens.	0.02	0.03	0.3	0.5	1.0	2.0

had been arbitrarily added to the media at the beginning of the experiments, with the purpose of gradually accustoming the organisms to the action of iodine and iodide. The doses given in the third to the sixth columns indicate the quantities added to the media where the spirochetes could still grow well. It may be stated here that these organisms, before passing through the iodinized media for some generations, could not grow in the presence of 0.7 cc. of Lugol's solution in 5 cc. of the culture media.

The color of Lugol's solution soon disappears from the media when the mixture is placed at 36°C. Even the addition of 2.5 cc. did not preserve the brown color over 48 hours. The most important factor affecting the antispirochetal action of the iodine solution was found to be the size of the fresh tissue added. The larger the tissue, the safer the spirochetes were from the inhibitory or sterilizing influence of the preparation. For this reason our experiments were carried out with as uniform a size of the tissue as practicable for each tube, and the results presented in Table VI may be considered approximately correct. It is true also with salvarsan, neosalvarsan, and bichloride of mercury, but the interference from this source was much less disturbing than with the iodine solution. Thus our figures in all the experiments should be taken as expressing merely the approximate values.

At the end of the experiments, a comparative study of the drug-fast and the ordinary stock cultures of the spirochetes in relation to their resistance to salvarsan, neosalvarsan, bichloride of mercury, and Lugol's solution was undertaken. The results are shown in Tables VII, VIII, IX, and X.

Attempts to carry out parallel experiments in solid media met with certain technical difficulties, in that the drugs had to be added while the mixture of the ascitic fluid and agar was still in fluid condition; that is, at a temperature of about 45°C. When salvarsan, neosalvarsan, and bichloride of mercury were mixed with the media, a distinct turbidity resulted, and solidification of the media at once followed, sometimes rendering a uniform distribution of the drug impossible. It was for this reason probably that we were unable to obtain results of definite constancy to warrant the assumption of the existence of an increased drug tolerance in the spirochetes when

TABLE VII.

Salvarsan.

In 5 cc. of media containing.	Strain McD., which grew in the tube containing 0.06 mg.	Strain McD., never in contact with salvarsan.
	Growth.	Growth.
mg.		
0.2	−	−
0.1	<+	−
0.05	+	−
0.01	++	+
0.005	++	++
No drug.	++	++

TABLE VIII.

Neosalvarsan.

In 5 cc. of media containing.	Strain R., which grew in the tube containing 0.1 mg.	Strain R., never in contact with neosalvarsan.
	Growth.	Growth.
mg.		
0.2	−	−
0.1	<+	−
0.05	<+	−
0.01	+	<+
0.005	++	<+
No drug.	++	++

TABLE IX.

Bichloride of Mercury.

In 5 cc. of media containing.	Strain Z. A., which grew in the tube containing 0.5 mg.	Strain Z. A., from original solid culture, never in mercuric chloride media.
	Growth.	Growth.
mg.		
1.0	−	−
0.5	<+	−
0.1	+	−
0.05	++	−
0.01	++	<+
No drug.	++	++

TABLE X.

Lugol's Solution.

In 5 cc. of media containing.	Strain McD., which grew in the tube containing 2 cc.	Strain McD., never in contact with Lugol's solution or iodide.
	Growth.	Growth.
cc.		
2.0	<+	−
1.0	+	−
0.5	++	±
0.1	++	+
0.05	++	+
No drug.	++	++

passed through the drug-containing media for several generations. Nor was it possible to demonstrate the acquired drug tolerance by means of solid media, which was easily seen in the fluid media. We are not prepared to offer an explanation for this phenomenon. Perhaps in fluid media the drugs enter into combination with certain tissue ingredients of the media and in this form induce a gradual tolerance on the part of the spirochetes, whereas with solid media the drugs are held in the agar in such a manner as not to become modified enough to produce increased resistance in the spirochetes.

With regard to the duration of the acquired drug tolerance of the spirochetes, a series of experiments was carried out in which the drugged strains were returned to the ordinary fluid media without any drug. From each successive generation, tolerance tests were made with varying concentrations. The results are summarized in Table XI.

As may be seen from Table XI, the acquired tolerance to various drugs decreases fairly rapidly. Within a period of 8 weeks, during which four transfers through ordinary media were made, the acquired tolerance of the *pallidum* and *refringens* to salvarsan and neosalvarsan entirely disappeared, and that of *microdentium* disappeared even sooner. The disappearance of the acquired tolerance to bichloride of mercury was more gradual, requiring about 10 weeks for complete restoration of the normal sensitiveness to this salt. In this respect, the paratyphoid strain used by Marks[2] differs greatly from the spirochetes, as the former passed through forty-six passages before returning to the initial tolerance.

TABLE XI.

Maximum Doses of Each Drug in Which Various Drug-Fast Strains of the Spirochetes Still Grew Well after Being Returned to Drug-Free Media for Successive Generations.

	Salvarsan.			Neosalvarsan.			Bichloride of mercury.		
	*T. pallidum.**	*T. microdentium.*	*S. refringens.*	*T. pallidum.**	*T. microdentium.*	*S. refringens.*	*T. pallidum.**	*T. microdentium.*	*S. refringens.*
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
After one generation in drug-free media....	0.1	0.05	0.05	0.15	0.1	0.1	0.5	0.1	0.1
" two generations in " "	0.05	0.03	0.03	0.1	0.05	0.08	0.3	0.1	0.1
" three " " " " ...	0.05	0.02	0.02	0.05	0.02	0.04	0.05	0.07	0.05
" four " " " "	0.03	0.01	0.02	0.03	0.02	0.03	0.04	0.04	0.03
" five " " " "	0.02	0.01	0.02	0.03	0.02	0.03	0.02	0.01	0.01
" six " " " "	0.02	0.01	0.02	0.03	0.02	0.03	0.02	0.01	0.01

* *Pallidum*, Strain McD.

SUMMARY.

In the foregoing experiments we attempted to determine whether or not, by subjecting several varieties of spirochetes to increasing doses of certain chemotherapeutic agents, a gradual increase of resistance to the latter could be shown. For this purpose, pure cultures of *Treponema pallidum*, *Treponema microdentium*, and *Spirochæta refringens* were used against the action of salvarsan, neosalvarsan, bichloride of mercury, and iodine-iodide potassium solution *in vitro*. For culture media, the usual ascites-broth-tissue medium as well as solid ascites-agar-tissue medium was used. After permitting the spirochetes to grow for a fortnight in media containing certain quantities of each drug, transfers were made from tubes showing various degrees of growth to the next series of tubes containing the same drug in still higher concentrations, and similar transfers repeated every 2 weeks. The results of the experiments may be briefly summarized as follows:

1. *Treponema pallidum* and *Treponema microdentium* have, within 3 to 4 months, increased their tolerance to salvarsan and neosalvarsan to five and one-half times their original mark. With *Spirochæta refringens* the increase was about three times.

2. Against the action of bichloride of mercury, the amount of increased tolerance of *Treponema pallidum* was about 35 to 70 times the original, while that of *Treponema microdentium* was about 10 times as much and was reached within 10 weeks. *Spirochæta refringens* resisted 30 times the original dose.

3. There was an unmistakable increase of resistance of these spirochetes to the action of the iodine-iodide solution (Lugol's solution) when they were grown for several generations in fluid media containing the iodine solution, but the rate of increase between the initial and the acquired tolerance was slight. In general, the addition of Lugol's solution to fluid media has a weak inhibitory influence upon the growth of the spirochetes, requiring for the total suppression of growth a quantity of over 0.7 cc. to 5 cc. of the culture media. The tolerance reached was for about three times that amount.

4. A similar tolerance phenomenon has not been established when employing a solid instead of a fluid medium containing the drugs. No explanation is offered except a suggestion that the drugs held in the agar do not enter into combination with certain tissue constituents of the medium as they are able to do with tissue elements in fluid media. This may be a factor necessary for inducing drug tolerance in these organisms *in vitro*.

5. The increased drug-fastness *in vitro* has a limit beyond which no further advance can be made. This limit varies with different species of spirochetes.

6. The acquired drug-fastness *in vitro* gradually disappears when the spirochetes are cultivated again in the drug-free media for several generations.