26. *Failure of Chicken Sarcoma to Reduce Methylene Blue, and its Relation to Lowered Oxygen Tension.*

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There seems to exist some fundamental peculiarities in the cells of the Rous chicken sarcoma. It was first shown by one of us (Nakahara)¹ that these cells are not always killed by desiccation, as are cells of other tissues, but appear to remain in the state of suspended animation for some time, reviving when placed under suitable conditions. It was suspected that such abnormal resistance to desiccation might conceivably be related to some peculiarities in the oxidation-reduction system. We later found that the chicken sarcoma contains an exceptionally minute amount of glutathione (Yaoi and Nakahara)², an autoxidizable substance of accepted importance to the respiration of living cells in general. The present paper has to do with further investigations, in which we believe to have demonstrated another striking fact, that this tumor is incapable of reducing methylene blue, in spite of the fact that it readily reduces the tension or oxygen *in vitro*.

Inability to reduce methylene blue.

In preliminary experiments the action on methylene blue of the fresh sarcoma tissue (Rous No. 1) was tested with the surprising result that the dye was not reduced as long as there was no bacterial contamination. The importance of the nature of this observation lead us to investigate the subject under better controlled experimental conditions.

Tissues to be tested were removed under aseptic precautions from freshly killed chickens, and were divided into 1 gram portions of two or three pieces each. These were placed in test tubes containing 10 cc. of phosphate saline solution, adjusted to P_H 7.6, and the whole was sterilized at 56°C for 30 minutes. After cooling, 0.5 cc. of sterile 0.1 per cent solution of methylene blue in distilled water was added, sealed with a 1 cm. layer of vaseline, and incubated at 37°C.

The results of repeated experiments showed that under the condi-

tion in which skeletal muscle completely reduced methylene blue within 16 to 20 hours, the Rous sarcoma failed to produce any reduction, though incubation was continued for 72 hours. Some discoloration occurred in the immediate vicinity of the tumor pieces, but it was due not to reduction but to absorption by the tissue, it being impossible to restore the blue color by blowing air into the faded dye.

Absence of thermostable reducing substance.

It is conceivable that the inability of the chicken sarcoma to reduce methylene blue may be related to the practical absence of glutathione. On the other hand, the negative result may be accounted for by the possible lack of the extractible, thermostable substance, known to be capable of strongly activating tissue respiration (Meyerhof's "Atmungskörper", 3. and Roelli's "Lösungsfaktor", 4). In order to determine this point, we next tested the action on methylene blue of boiled and filtered extracts in combination with various extracted tissues.

Extract was prepared by boiling for one hour 1 gram of fresh, finely chopped tissue with 5 cc. of saline solution in the autoclave, filtering, adjusting the reaction with N. NaOH to P_H 7.6, and boiling again until it completely lost the nitroprusside reaction and became incapable by itself of reducing methylene blue. It was used for test within 24 hours. The extracted boiled tissue was strained through a seive, and was washed in water repeatedly, until unaided it no longer showed nitroprusside reaction or reducing action on methylene blue. 1 gram of extracted tissue and 5 cc. of boiled extract were placed in a test tube with sufficient phosphate saline solution to make the total volume of 10 cc. The reaction of the mixture was re-adjusted to P_H 7.6. The tube was then immersed in boiling water, and the result read after 30 minutes. This method proved to be much more rapid and satisfactory than the ordinary technique of evacuating the tube and placing it in the anaerobic jar at a constant temperature. The discoloration due to absorption by the tissue was carefully ruled out by testing the reversibility of the color change through contact with air. All the experiments were carried out in duplicate.

The results, as described below, leave little doubt that the chicken sarcoma is lacking in the extractible, thermostable reducing substance.

Table 1.—Reduction of methylene blue.

Liver or muscle alone	—
Liver or muscle extract alone	—

Liver or muscle <i>plus liver or muscle extract</i>	+
Sarcoma alone	—
Sarcoma extract alone	—
Sarcoma <i>plus sarcoma extract</i>	—
Liver or muscle <i>plus sarcoma extract</i>	—
Sarcoma <i>plus liver or muscle extract</i>	+

*Lowered oxygen tension evidenced by the growth
of anaerobic organisms.*

The above results raise an interesting question as to the well known parallelism existing between the reduction of methylene blue and lowered oxygen tension. Hall (5), Gates and Olitsky (6), among others, accepted the reduction of methylene blue as an indication of the cultural condition which permits the growth of anaerobic microorganisms. We, therefore, attempted to determine if the chicken sarcoma, incapable of reducing methylene blue, can reduce the tension of oxygen by testing the utilizability of this tissue for the cultivation of some anaerobic organisms.

Our experiments showed that *Treponema pallidum* can be cultivated in modified Noguchi's media in which the piece of rabbit's kidney is replaced by that of chicken sarcoma (Table 2), and we successfully passed *Treponema pallidum* through three generations in the media containing sarcoma tissue.

Table 2.—Growth of *Spirochaeta pallida*.

	Rabbit kidney media.					Chicken sarcoma media.				
	Tube No. 1	2	3	4	5	Tube No. 1	2	3	4	5
10 days	+	+	+	+	+	+	+	+	+	+
15 days	+	++	++	++	++	+	+++	+++	++	++
20 days	+++	+++	+++	+++	+++	++	+++	+++	++	+++

We also found that another anaerobic organism, *Clostridium tetani*, can be readily cultivated in suitable media containing 1 gram of extracted sarcoma or other tissue with 10 cc. of boiled sarcoma extract, in spite of the fact that these combinations do not reduce methylene blue, as already stated. The growth of *C. tetani* under these conditions was as good as, or better than, in the case in which the media contained a combination of tissues and extracts which rapidly reduced methylene blue (liver or muscle plus liver or muscle extract), and second generation cultures were also easily obtained. All these experiments were made in duplicate.

Table 3.—Growth of *Clostridium tetani*.

	1st generation (48 hrs.)	2nd generation (48 hrs.)
Liver plus liver extract	+++	+++

Muscle plus muscle extract	+	—
Sarcoma plus sarcoma extract	+++	+++
Liver plus sarcoma extract	+++	+++
Muscle plus sarcoma extract	++	++

It is of interest to add that an aerobic organism, *Leptospira icterohaemorrhagiae*, failed absolutely to grow when a piece of the sarcoma tissue was added to the medium (Takaki's) which is otherwise suitable for the growth of this species. This inability to grow on the part of the aerobic organism furnishes additional evidence of the reduction of oxygen tension by the sarcoma tissue.

We purposely avoid a discussion of various theories regarding the mechanism of oxidation and reduction, and the significance of tissues in the cultivation of anaerobic microorganisms, though our observations suggest certain objections to some of the contemporary ideas. We shall consider these points in our further investigations.

Conclusions.

The tissue of the Rous chicken sarcoma is lacking in the extractible, thermostable substance which brings about the reduction of methylene blue. In spite of this exceptional condition, this tissue placed in culture media sufficiently lowers the tension of oxygen to permit profuse growth of anaerobic microorganisms, such as *Treponema pallidum* and *Clostridium tetani*. These facts clearly demonstrate that the reduction of methylene blue is not necessarily related to the lowered oxygen tension, as hitherto generally accepted, and offer an important material for the study of the oxidation-reduction system of tissues.

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