controls were predominantly necrotic exudative. The lesions in the treated animals were extensive, occupied almost all alveoli, but the numbers of tubercle bacilli were considerably reduced as compared with the controls. The livers and spleens of such animals were extremely congested and this, in combination with the great reduction in space for gas exchange in the lungs, suggests that death may have resulted primarily from anoxia and right heart failure rather than from the toxic effects of the tuberculous infection.

These findings indicate that mice may be benefited even late in the course of tuberculosis induced by intravenous inoculation. All groups, whether treated from the day of inoculation or from the 7th, 14th, or 21st days had the same amount of disease on death, and since the average survival times were not significantly different, it would seem that the subjects with most severe disease at the time treatment was started must have obtained relatively greater benefit from streptomycin. The explanation for this phenomenon, however, is not readily apparent.

*Conclusion.* Streptomycin to the amount of 3000 μg per day had a favorable effect upon the course of experimental tuberculosis in mice when treatment was initiated 7, 14 and 21 days following infection.

16588

## A New Chromosome Stain and Its Relationship to Atypical Cell Proliferation.

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The metabolic transformations of paraphenylenediamine and certain azo-dyes into quinone diimine (Mayer[1]) are accompanied by staining effects on epidermal and other cells. This would indicate that quinone diimine possesses a strong affinity for certain nuclear material to which it becomes attached. Since it seemed probable that the cell proliferations with precancerous and cancerous degeneration produced by aromatic amines are caused by the affinity of their oxidation products for certain vital components of the cell nuclei, we have investigated that part of the nucleus with which quinone diimine combines. We have therefore attempted to stain chromosomes with the oxidation products of paraphenylenediamine. and several of its derivatives.

Certain substitution products of paraphenylenediamine, such as dimethyl- and tetramethyl paraphenylenediamine and also metaphenylenediamine, have been used as microscopic or vital stains by Wurster,[2,3] Unna[4] and Joseph and Wurster.[5] In combination with β-naphthol, dimethyl paraphenylenediamine forms blue indophenol in the presence of oxidative cell enzymes (Ehrlich[6]), a reaction known as the "Nadi reaction". No reference in the literature has been found on similar uses of paraphenylenediamine itself.

*Methods. Source of chromosomes.* Salivary glands of *Drosophila melanogaster* and *Drosophila robusta* larvae* maintained on molasses agar were prepared in the usual

1 Mayer, R. L., *J. Invest. Dermatol.*, 1948, **10**, 389.

2 Wurster, C., *Ber. Dtsch. Chem. Ges.*, 1886, **19**, 3195.

3 Wurster, C., *Ber. Dtsch. Chem. Ges.*, 1887, **20**, 256.

4 Unna, P. G., *Monatsh. Prakt. Dermat.*, 1887, **6**, 62.

5 Joseph, M., and Wurster, C., *Monatsh. Prakt. Dermat.*, 1887, **6**, 243.

6 Ehrlich, P., *Das Sauerstoffbedurfnis des Organismus*, Berlin, 1885.

way (Darlington and LaCour[7]). The isolated glands were suspended on a microscope slide in a drop of 50% acetic acid.

*Staining solutions prepared from paraphenylenediamines.* 0.2 g of paraphenylenediamine or its derivatives, namely *s*-dimethyl paraphenylenediamine dioxalate and tetramethyl paraphenylenediamine hydrochloride were dissolved in 20 ml of hot 20% acetic acid. 0.1 ml of 30% hydrogen peroxide was added and the mixture cooled as soon as a light-brown color had developed. The oxidation continues slowly and after approximately 30 minutes the stain is ready to use. The solutions of paraphenylenediamine are then dark-brown and those of the 2 derivatives red and purple respectively.

Another staining solution prepared from pure quinone compounds is described under staining process, part b.

*Staining process.* a). *Stain prepared from aqueous solutions of paraphenylenediamine.* This stain is very sensitive to certain metals; traces of iron, dissolved from dissecting needles in contact with acetic acid, suffice to rapidly produce dark-purple or brown colorations and precipitations. Such an iron-containing stain provides excellent specimens, but has the disadvantage that the protoplasm and connective tissue stain more darkly than in absence of iron. It is therefore preferable to avoid contamination with iron by removing the acetic acid solution, in which the salivary glands are suspended and which had been in contact with the dissecting needles with filter paper before adding the stain. The slides are stored for one hour in Petri dishes over moist filter paper, then covered with a cover glass and squash preparations made in the usual way. If the coloration is too light, another drop of the staining solution may be placed under the cover glass for an additional 30 minutes.

Particularly good preparations were obtained when the glands were pulped in the acetic acid solution before staining.

b). *Stain prepared with pure quinone diimine.* Since the oxidation of paraphenylenediamine with hydrogen peroxide in water furnishes a large number of darkly colored intermediate and polymerization products, pure quinone diimine was prepared according to Willstaedter and Pfannenstiel.[8] Freshly precipitated silver oxide is thoroughly washed with water, followed by anhydrous acetone and ether to remove all traces of salts and water and then suspended in absolute ether. 0.2 g of paraphenylenediamine base are then dissolved in 100 ml of absolute ether, 1.0 g of the water-free silver oxide added and the mixture shaken for 2 hours. After filtration the ether is removed on a water bath. The yellow to reddish crystals remaining are very unstable, and the quinone diimine crystals must be freshly prepared for each staining series. The following procedure gives the best results: Salivary glands are suspended in 50% acetic acid. Ten mg of the quinone diimine crystals are dissolved in 1 cc of 70% acetic acid; the solution turns dark-blue with formation of a deeply-colored precipitate, which dissolves in an excess of acetic acid. A weakly-colored solution results which may be added immediately to the chromosomes placed on the slide; the development of the stain in this case requires 10 to 20 minutes.

Instead of isolating the quinone diimine from the solvent, the ether solution of the quinone diimine may be used directly for staining. Salivary glands are suspended in 50% acetic acid, crushed preparations made and the material re-suspended in a drop of 70% acetic acid. A drop of the ether solution containing the quinone diimine as prepared according to Willstaedter[8] is then added. Again, immediately upon contact with the acetic acid solution the quinone diimine is transformed into a deep-blue compound which re-dissolves immediately in excess of acid to yield a light-brownish color. About one-half hour later the chromosomes are very dark, purple-brownish, whereas the protoplasm in

* We wish to thank Dr. Max Levitan, Columbia University, for the strain of *Drosophila robusta* as well as for much valuable technical information.

7 Darlington, C. D., and La Cour, L. F., *The Handling of Chromosomes*, 2nd Edition, London, 1947.

8 Willstaedter, R., and Pfannenstiel, A., *Ber. Dtsch. Chem. Ges.*, 1904, **37**, 4605.

most instances remains almost uncolored. This procedure has not only given the best results, but has also prevented the formation of precipitates on the slides.

From the practical standpoint, however, the staining procedure in which aqueous solutions of paraphenylenediamine are oxidized with hydrogen peroxide and the resulting solution used as stain, is the simplest and provides specimens of sufficient contrast for most purposes.

c). *Stains prepared from s-dimethyl- and tetramethyl paraphenylenediamine, quinone chlorimine and quinone dichlorimine.* The dimethyl and tetramethyl derivatives of paraphenylenediamine oxidized in water with $H_2O_2$ as described above and applied to the salivary glands under the same conditions as oxidized paraphenylenediamine or quinone diimine, failed to stain the nuclei and chromosomes. Similar negative results were regularly obtained using pure quinone chlorimine and pure quinone dichlorimine. In either case the protoplasm became distinctly colored and showed the sites of the nuclei as uncolored areas. The substituted quinone diimines have a much lower basicity than unsubstituted quinone diimine, which apparently prevents their combination with nuclear material and favors the combination with protoplasm protein.

d). *The staining of chromosomes at pH 6.5.* It is possible to stain chromosomes as well as nucleoli with 1 to 10% paraphenylenediamine diacetate dissolved in pH 6.5 acetate buffer. The purple stain, which develops upon exposure to air within several hours, is more diffuse than when made at lower pH values.

*Microscopic aspect.* In properly stained preparations the nuclei appear as dark-brown spots on a yellowish-brown background. The chromosomes disclose a typical succession of broader or narrower colored and uncolored bands and granulations, the color of these bands or granules ranging from brown to deep-black, often with a definite purple component (Fig. 1 and 2).

Contrary to the chromosome specimens stained with aceto-carmine or aceto-orcein which fade within a short time, the color of specimens stained with quinone diimine is stable for many weeks. Often the color further deepens on standing.

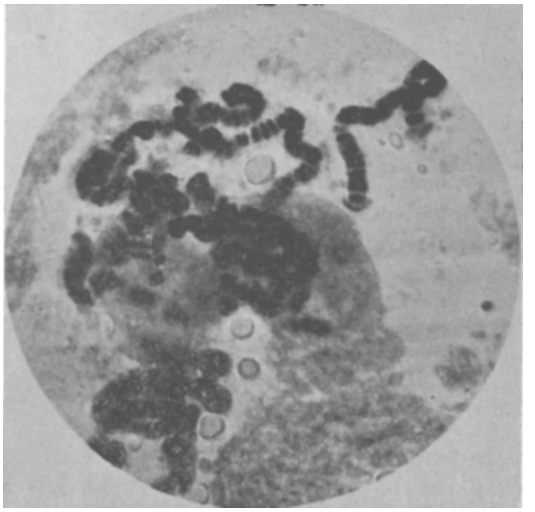

FIG. 1.
Chromosomes stained with oxidized paraphenylenediamine.

*Discussion.* Specimens of chromosomes stained with quinone diimine very closely resemble, in their general aspect, chromosomes stained with the usual chromosome dyes. Similarly, a comparison of the distribution of the bands in specimens stained by the various staining methods strongly suggests that the quinone diimine stain affects the same areas, and, consequently, the same components of the chromosomes which are stained with aceto-carmine, aceto-orcein, or the Feulgen technic.

Considering the chemical reactions characteristic for quinone diimine and other compounds of quinone structure, we believe that the quinone diimine chromosome stain is based upon the formation on an anilido quinone-like fixation of quinone diimine to the nucleoproteids of the chromosomes, *i.e.* the genes.

Previous experiments[1] have suggested that a combination of compounds of quinone structure, especially quinone diimine, with proteins and nucleoproteids takes place within the living body when certain aromatic amines are introduced and there produce pathological reactions.

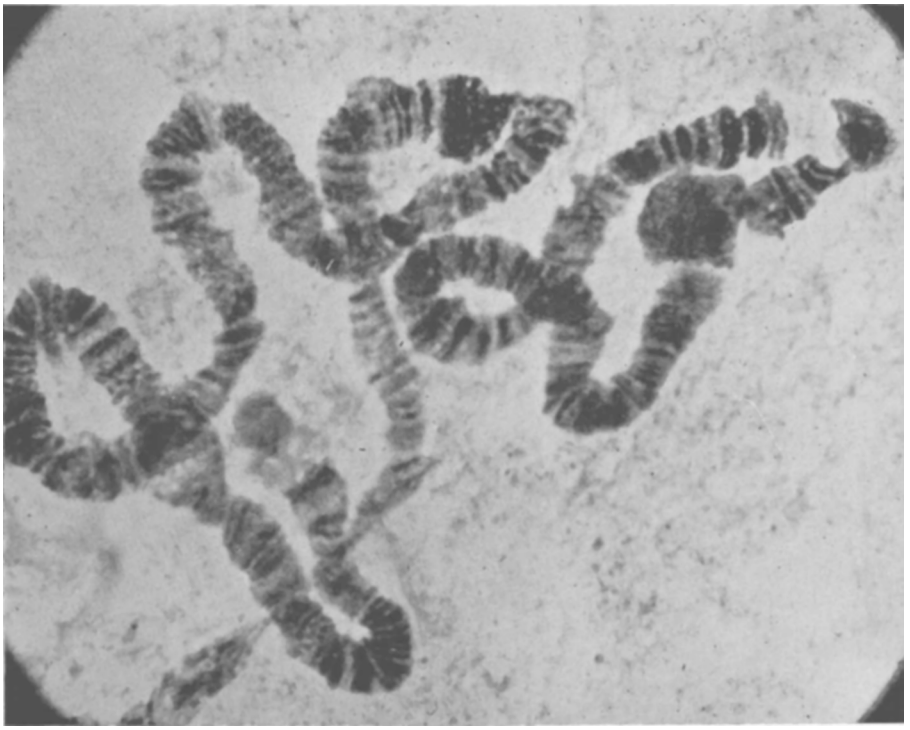

FIG. 2.
Chromosomes stained with quinone diimine.

It is known from clinical experiences and animal investigations that aromatic amines such as paraphenylenediamine, naphthylamine and certain azo-dyes containing aromatic amino groups such as butter yellow and scarlet red produce allergic sensitizations—asthma and dermatitis—and atypical epithelial proliferations (Fischer,[9,10] Sachs,[11] Martinotti[12]), as well as cancer (Yamagiwa and Ohno,[13] Hueper,[14,15,16] Sasaki and Yoshida,[17] Yoshida[18,19] Miller and co-workers[20] and Harris and co-workers[21]). There are many indications that these amines are only the precursors of the directly active principle which is formed within the body after a series of metabolic transformations.

Azo-dyes, serving as hydrogen acceptors, are reduced to amines and diamines, especially paraphenylenediamine, which are then oxidized into quinone diimine and related compounds of quinone structure (Mayer[22-25]). Aromatic amines or paraphenylenediamine

9 Fischer, B., *Munch. Med. Wschr.*, 1906, **53**, 2041.

10 Fischer, B., *Frankf. Z. Path.*, 1922, **27**, 98.

11 Sachs, O., *Arch. f. Dermat.*, 1913, **116**, 559.

12 Martinotti, *Berl. Klin. Wschr.*, 1914, 1441.

13 Yamagiwa, K., and Ohno, S., *Jap. Z. f. Krebsf.*, 1918, **12**, 3.

14 Hueper, W. C., Willy, F. H., and Wolfe, H. D., *J. Indust. Hyg. and Toxicol.*, 1938, **20**, 26 and 85.

15 Hueper, W. C., *Arch. Path.*, 1938, **25**, 856.

16 Hueper, W. C., Briggs, F. A., and Wolfe, H. D., *J. Indust. Hyg. and Toxicol.*, 1938, **20**, 85.

17 Sasaki, T., and Yoshida, T., *Virchows Arch. Path. Anat.*, 1935, **295**, 175.

18 Yoshida, T., *Transact. Japan. Path. Soc.*, 1933, **23**, 636.

19 Yoshida, T., *Transact. Japan. Path. Soc.*, 1934, **24**, 523.

20 Miller, J. A., Kline, B. E., Rusch, H. P., and Baumann, C. A., *Cancer Research*, 1944, **4**, 153.

21 Harris, M., Krahl, M. E., and Clowes, G. H. A., *Cancer Research*, 1947, **7**, 162.

22 Mayer, R. L., *Arch. f. Dermatol.*, 1928, **156**, 331.

23 Mayer, R. L., *Arch. f. Dermatol.*, 1929, **158**, 266.

are directly oxidized. The first phase of these metabolic transformations, namely the reduction of azo-dyes to paraphenylenediamine, has been studied by Nitti and coworkers[26] and by Stevenson, Dobriner and Rhoads.[27] An interesting example of a biological oxidation of an aromatic amine associated with the production of cancer has been described by Haddow.[28]

Contrary to the slowly reacting amines and azo-dyes, most quinone derivatives are biologically very active, being strong oxidizing agents. However, the property in which we are especially interested is their ability to combine with numerous aromatic and aliphatic compounds to form anilido quinones or anilido quinone imines (Fischer,[29] Posner,[30] Martynoff and Tsatsas,[31] Suida and Suida[32]). Among the body constituents with which quinone diimine readily combines are various amino acids, especially those which contain -S-S or SH groups, proteins and nucleoproteids.

We have explained the high allergic activity of paraphenylenediamine as the result of the formation of anilido quinone-like combination with body proteins, and the production of atypical cell proliferation with the formation of similar combinations with nucleoproteids (Mayer[1,22]).

As the present study on chromosome stains suggests, an anilido quinone-like fixation of metabolically formed quinone diimine to chromosomes or genes may readily take place when sufficient amounts of aromatic amines or azodyes are introduced into the body. This combination with the specific material of the chromosomes is very strong, the chromosome stain resisting a great number of chemical influences; it is strong enough to account for disturbances in the physiological behavior of the chromosomes and genes.

Further experiments are necessary to prove that it really is such a blockade of the chromosomes and the combination of genes with quinone derivatives as a consequence of contact with aromatic amines which leads to atypical proliferations of the affected cells and, in certain cases, even to mutations and malignant degeneration.

Besides its general interest with respect to the problem of atypical cell proliferations and cancer, the quinone diimine stain possesses certain technical advantages over the known chromosome stains. In many instances the chromosomes made visible by our procedure are more distinct than with other stains; moreover, in varying the time of exposure to the stain it is possible to obtain wide variations in the intensity of coloration, thus enhancing or suppressing certain features.

*Conclusion.* A new chromosome stain is described which is based upon the affinity of nucleoproteids for certain quinone derivatives. The relationship between this stain and the production of atypical epithelial proliferations and cancer produced by aromatic amines is discussed.

Acknowledgment is made to Miss Jeanne Long, Parasitology Laboratory, for maintaining the strains of Drosophila.

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[28] Haddow, A., *Brit. Med. Bull.*, 1947, **4**, 1.

[29] Fischer, E., and Schrader, H., *Ber. Dtsch. Chem. Ges.*, 1910, **43**, 525.

[30] Posner, T., *Liebigs Ann.*, 1904, **336**, 108.

[31] Martynoff, M., and Tsatsas, G., *Bull. Soc. Chim. France*, 1947, 52.

[32] Suida, H., and Suida, W., *Liebigs Ann.*, 1918, **416**, 113.