Technical Papers

Reversal of Gram-staining Behavior

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In a recent article, Bartholomew and Mittwer (*1*) report the conversion of gram-positive organisms to gram-negative ones by ultraviolet irradiation. In two recent papers (*2, 3*) we proposed a mechanism for the gram-staining reversal and showed that gram-positive bacteria, as well as alkali-treated wool, could be converted to a gram-negative state by acids or by oxidizing agents and could be reversed back to the gram-positive state by means of alkalis or by reducing agents.

Bartholomew and Mittwer's results appear at first sight to be contrary to the view expressed in our discussion of the possible role of ultraviolet light on the gram-staining reversal (*2*). On further study, however, the results of Bartholomew and Mittwer actually bring added evidence for the mechanism postulated. Meunier (*4*) has shown that the action of light on wool is to make the sulfur more labile and to convert the cystine sulfur to sulfur dioxide and finally to the trioxide, which is freed in the form of sulfuric acid. The experiments of Smith and Harris (*5*) also indicate the formation of sulfate by the photochemical oxidation of wool. Moreover, this was shown to be accelerated by the presence of acids. The formation of hydrogen sulfide appears to be the first step in this process. The protective action of formaldehyde on the degradation of proteins, and of wool in particular, is well known.

It thus seems that the action of reducing agents and of oxidizing agents in the gram-staining mechanism which we postulated can also explain the results of Bartholomew and Mittwer. The action of ultraviolet radiation is probably the following. The first step is a reducing or hydrolytic one whereby —SH and —SOH groups are formed from the cystine linkages. This was the reason for supposing that gram-negative organisms would be converted to gram-positive ones. Mirsky and Anson (*6*) found also that ultraviolet light liberates —SH groups in many proteins. This first step might explain why the formation of gram-negative cells observed by Bartholomew and Mittwer was slow at first and seemed to require an induction period. However, the initial formation of —SH groups is superseded by an oxidation which results in gram-negative response, as we have already postulated. Since formaldehyde has a protective action and a reducing one, its retarding effect on the conversion noted by Bartholomew and Mittwer is readily understood. On the other hand, osmic acid, being oxidizing, would hasten the reaction as observed by Bartholomew and Mittwer. The accelerating effect of acids noted by Smith and Harris (*5*) should be recalled in this connection.

Thus the changes in gram-staining behavior mentioned above emphasize once more the analogy between the behavior of the cytoplasmic membrane of gram-negative bacteria and that of untreated wool, as well as that between gram-positive bacteria and alkali-treated wool (*2*). The role of the ribonucleic acid in the gram stain reversal was also discussed in the above-cited communication.

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Manuscript received August 25, 1952.

Formation of a Labile Pigment in Rabbit Ova During Histochemical Demonstration of Succinic Dehydrogenase[1]

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The histochemical demonstration of succinic dehydrogenase, one of the vital respiratory enzymes, has been previously described (*1–3*). The reaction is based on the reduction of a tetrazolium salt during incubation of fresh tissue in the presence of an excess of sodium succinate (*1*). If neotetrazolium (*pp'*-diphenylene bis 2-(3,5 diphenyl tetrazolium chloride)) (NT) is employed, the reduction of NT is not reversible (*4*). The reduction compound is seen as fine black granules in the cells.

Previously, we demonstrated succinic dehydrogenase activity in ovaries of rabbits injected with urine from pregnant or nonpregnant women (*3*). Fresh 3-mm blocks were incubated for 1 hr at 37° C in 0.9% NT in normal saline with 0.1 *M* phosphate, buffered to pH 7.4 with the addition of 0.03 *M* sodium succinate. The blocks were fixed in formalin neutralized with magnesium carbonate. Twenty-four hr later, frozen sections (15 μ) were cut from the blocks. In the 18 rabbits reported in this series (*3*), as well as in 4 other rabbits separately studied, little or no black pigment

[1] This investigation was supported by a research grant C-1486 from the National Cancer Institute, of the National Institutes of Health, USPHS.

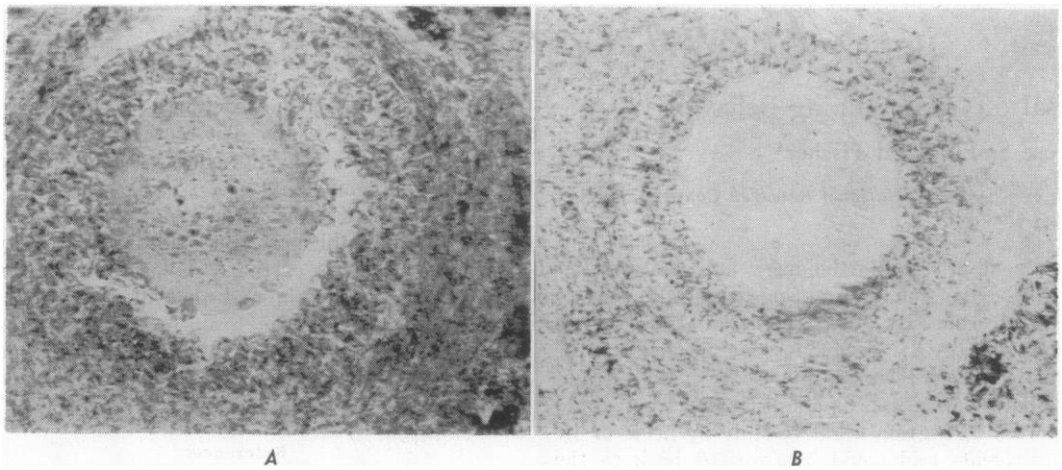

FIG. 1. Frozen section (15 μ) of rabbit ovary incubated in NT with succinate. Succinic dehydrogenase demonstrated by deposition of pigment. × 600. *A*, section prepared 24 hr after incubation, with granular black pigment in follicle cells, light staining of zona pellucida, and microdroplets in ovum. *B*, section from same block after 4 weeks' storage in formalin. Little change in distribution pattern of granular black pigment. Ovum and zona pellucida are now colorless.

was seen in the ova, whereas the adjacent follicle cells contained appreciable quantities of black granules. In addition, a reddish pigment was found in varying quantities through the ovaries, interspersed with the black pigment granules. Only in the ova, however, was this reddish pigment found alone. Here it was seen as microdroplets of varying size, rather than the granule or crystal formation suggested by the black reduced NT in other portions of the ovaries. When additional sections were cut from the same ovaries 4 weeks later (in part as a control procedure) all reddish pigment, including that from the ova, had disappeared. The black pigment of reduced NT seemed unchanged in amount and distribution.

As a further investigation, additional blocks of ovaries of 3 rabbits receiving no urine injections were similarly processed in NT with succinate. All blocks were fixed in formalin neutralized with magnesium carbonate having a pH of 9.2. In the frozen sections cut 24 hr later, black granules, some resembling short needlelike crystals (*2*), were found in follicle cells, in cystic follicles, and in the stromal cells, as previously described (*3*). Virtually no black granules were found in the ova. A less prominent reddish pigment deposition was mingled with the black granule deposits. The reddish droplets were also found in moderate quantity in the ova (Fig. 1 *A*) with light pink staining of the zona pellucida. After 4 weeks, the mean pH of the fixative had declined to 7.7. Additional frozen sections from the blocks at this time showed complete disappearance of all reddish pigment, including that seen in the ova (Fig. 1 *B*). The ova were colorless. The black pigment granules retained the previous pattern.

It is possible that a certain element of this reddish staining may represent dissolved reduced NT in the abundant lipoidal content of the ovary. This type of staining of fat during dehydrogenase demonstration has been reported by Seligman and Rutenburg (*1*). We have observed such lipoidal staining in hilar fat attached to ovaries being incubated in NT with succinate. This staining also disappeared on storage in formalin. It is our impression, however, that, at least in part, the formation of reddish pigment in the ova in the current study is an incomplete reduction compound of NT, based on the following:

1. In an analogous situation, Seligman and Rutenburg reported formation of a reddish-purple color in areas where enzymatic activity was low, by partial reduction of a tetrazolium salt to a monoformazan. They suggested that the reduction potential may have been lower than in areas stained blue (*1*).
2. The reddish staining of hilar adipose tissue attached to ovaries being processed in NT with succinate resulted in a diffuse reddish coloration, rather than the microdroplet formation found in the present study.
3. After formalin fixation, the ovaries from 7 rabbits, as well as human and rabbit adipose tissue in the current study, did not respond in any manner to incubation in NT with succinate (*1*).
4. In our experience, use of this technique with preparation of frozen sections 24 hr after initial processing in NT has resulted in admixture of small amounts of reddish-brown pigment with the blue-black formazan in various areas of 100 cervical biopsies, including regions not commonly considered to contain stainable lipoid (*5*).

The evanescence of the reddish pigment suggests the formation of a different chemical compound in this phase of the reaction. It does not seem related to disappearance of fat, since after the 4 weeks' storage, frozen sections from the ovaries in the current series showed abundant reaction in the ova to a conventional fat stain such as scarlet red. Reduced NT, in the form of blue-black pigment formation, showed little change in pattern with the passage of time, in our studies. The reduction of NT to the purple to black compound was not considered reversible by Antopol and co-workers (*4*).

It is reasonable to assume that the presence of the

labile reddish pigment within the rabbit ova as the sole manifestation of succinic dehydrogenase may signify the presence of a localized lesser degree of activity of this enzyme than found in certain other components of the ovary.

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Manuscript received September 11, 1952.

Paper Chromatography of Corticosteroids

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The paper chromatography of corticosteroids has been reported by other workers (*1–3*) and has generally given rise to reliable methods for the identification of such of these steroids as are known compounds. The present communication relates to a procedure for the paper chromatography of corticosteroids, in which a number of the usual techniques employed are simpler and more rapid than those previously described.

Although the present procedure is applicable to both descending and ascending paper chromatography, it has been more frequently applied to the latter and will be described as such. A sheet of Whatman No. 1 filter paper (43 × 43 cm) is folded into a cylinder in the manner indicated by Wolfson *et al.* (*4*). The materials to be chromatographed are applied to the paper as droplets of the appropriate solutions at points about 2 cm apart on the starting line, the latter being 8 cm from the bottom fold of the paper, and the spots formed by the droplets being not greater than about 0.5 cm in diameter. The paper is then placed in a cylindrical glass jar 15 cm in diameter and 46 cm high (Fisher Scientific Co., New York) containing a one-phase solvent mixture of xylene (225 ml) and absolute methanol (75 ml), and the jar is subsequently closed by means of a gastight cover. The solvent is allowed to ascend on the paper to a distance of about 25 cm from the starting line—this occurs over a period of about 2.5 hr. The paper is then removed from the jar and air-dried. Visualization of the locations of the various steroids and their quantitative estimation may then be performed by any of the known procedures (*1–3, 5–8*).

The R_f values obtained with a number of authentic corticosteroids[1] are shown in Table 1. It will be observed that drying the paper prior to chromatography results in a change of the R_f values. In general the results obtained with predried paper were found to be more reproducible, predrying presumably minimizing partition phenomena involving the moisture that may otherwise be present on the paper.

Whenever the quantities of materials applied to the starting line on the paper are equivalent to about 5 μg of standard corticosteroid, there is obtained, after development and visualization of the location of substances, a number of discrete spots corresponding to the various steroids present. Approximate dimensions of the various spots obtained are included in Table 1.

Advantages of the above procedure over those reported in the literature are as follows: A one-phase solvent system is used throughout, rendering unnecessary the equilibration of the paper in the solvent vapors prior to development; pretreatment of the paper with any solvent is unnecessary; lateral diffusion of steroid spots during development is quite limited, thus rendering unnecessary the precutting of the paper into a pattern of separated strips; the development period required for the resolution of mixtures of corticosteroids is not greater than 2–3 hr; air-drying of the paper afterwards is completed in less than 30 min.

TABLE 1

Corticosteroid		Paper dried 15 min at 100° C prior to development		Paper not dried prior to development	
Chemical Name	Letter Designation	R_f	Spot dimensions (horizontal × vertical diameter) cm	R_f	Spot dimensions (horizontal × vertical diameter) cm
11-Desoxycorticosterone	Q (Reichstein)	0.921	0.9 × 1.0	0.826	0.8 × 1.7
11-Desoxy-17α-hydroxycorticosterone	S "	.698	.7 × 5.5	.495	.9 × 3.8
Corticosterone	B (Kendall)	.653	.6 × 4.7	.491	.9 × 2.9
11-Dehydro-17α-hydroxycorticosterone	E "	.501	.8 × 3.5	.394	0.7 × 0.9
17α-Hydroxycorticosterone	F "	0.362	0.7 × 3.5	0.312	1.1 × 1.3

As in the case of the previously reported procedures for the paper chromatography of corticosteroids, the R_f values obtained by the present procedure are relative rather than absolute. Further, it is considered essential to base the final identification of an unknown corticosteroid on a direct comparison with authentic specimens of known substances, employing such criteria as specific color reactions (*6, 7*) and absorption spectra of sulfuric acid chromogens (*8*).

[1] Samples of corticosterone and 17α-hydroxycorticosterone were generously donated by Carl Djerassi and Gregory Pincus, and were kindly obtained for us by B. J. Brent.