The Selectivity of the Gram-stain for Keratins

The keratin structure of wool or hair is well known to be present in skin epithelium, feathers, myosin, actin, fibrinogen, nails, horn, haemoglobin, vitelline and shell membrane of the egg as well as in dental enamel. Recently it was shown that bacterial membranes[1] and the membrane sheath of spermatozoa[2] also behave like wool-keratin.

In spite of such a wide variety of distribution, no specific or selective staining method seems to have been found for keratins. Neither MASSON's trichrome stain in MALLORY's adaptation as recommended by CONN[3] for the "staining of keratin", nor the Gram-stain of ERNST[4] claimed as "nicht restlos spezifisch" by ROULET[5] apparently fulfill the desired criteria.

Since some of the previous work of the author was concerned with the mechanism of Gram-stain reversal[6] of keratins present in wool or in bacteria, it was decided to investigate the selectivity and limitations of the Gram-stain, especially in histological specimens containing keratins.

In studying the keratin parts of normal and malignant epithelial sections, the results confirmed the very old and seemingly forgotten observations of ERNST[4], who detected the selective action of the Gram-stain for certain keratins. However ERNST in his time could not understand why the "fertige Haarschaft", that is the fully developed or keratinized hair did not take the dye when stained according to GRAM. He was not aware of the change that takes place in the composition of the hair-keratin as it is formed in the follicle. We now know that most of the sulphur in the hair itself is in the form of cystine (containing –S–S– bridges) whereas in the intrafollicular part of the hair the sulphur is largely in the form of SH-groupings. Thus fully keratinized hair, nails, etc. stain Gram-negatively, whereas similar structures, but only partially developed, e.g. hair in the process of growth in the follicle, stain Gram-positively; the SH-groups present in this preformative eukeratin-stage are similar to those present in fully developed α-keratins which had been treated (degraded) with alkali or reducing agents[1]. We conclude therefore that the presence of SH- and other reducing groups in the keratins of wool, hair, or nails is associated with the Gram-positive staining behavior.

The Gram-negativeness of intact wool[7] or that of Gram-negative bacteria[8] could be reversed to a Gram-positive staining behavior by treating them with alkalis or reducing agents. Alkali-degraded wool or Gram-positive bacteria on the other hand can be made to behave Gram-negatively if treated with acids or oxidizing agents prior the Gram-staining procedure[9]. Contrary to the results with wools, conversion experiments with keratin derived from either benign or malignant stratified squamous epithelium did not alter the Gram-positiveness of the keratin. The experiment was attempted by treating the keratin for 5 to 10 h with a neutralized or even slightly alkaline 1% solution of sodium metabisulphite. In normal skin the stratum corneum of the surface epithelium is strongly Gram-positive and the keratohyalin granules of the stratum granulosum are slightly so. It again proved impossible to reverse this staining behavior by treating such material with 0·5% aqueous picric acid solution for 24 h at room temperature. These results are summarized and tabulated with some other characteristic features of keratins[1] in the Table.

As may be seen in the Table, the hard keratins which are present in wool, etc. are characterized by a reversibility of their Gram-staining behavior, whereas the soft keratins which are present in the epidermis (and in nervous tissue) are characterized by irreversibility of this behavior. This fundamental difference is correlated with a difference in chemical composition as indicated in the Table.

The only other commonly occurring protein which might show reversibility of Gram-staining behavior similar to that of the hard keratins is insulin. It also has a high cystine content (12·5%)[2].

The sulphur content (designated as A-type) of the soft keratins is low, whereas that of the hard keratins is high (A + B-type), and at first sight the difference in reversibility of the Gram-staining behavior might be thought to be correlated with this difference in sulphur content. However intact wool when degraded and thereby renderes Gram-positive, loses considerable amounts of its A-type sulphur[3] but in spite of this loss can again be rendered Gram negative according to the scheme mentioned above[4]. Thus it is apparent that the reversibility or non-reversibility of Gram-staining behavior in the keratins is dependent not so much on the quantity of sulphur present as upon the presence of a different sulphur fraction. This B-type sulphur fraction, mainly cystine bound differently[5] gives rise to reducing acidic groups and might be involved also in increased basophilia if the wool is exposed to mild alkaline agents[6]. The B-sulphur fraction is not present in epidermal keratin, a fact which is revealed by irreversibility of its Gram-positiveness. The sulphur in epidermal keratin might be present in fairly stable linkages, probably similar to those present in "overdegraded" wools, which are deprived of their B-type of sulphur and thus can no longer be reversed to a Gram-negative behavior. The stable type of linkage to which we refer is – C– S– C– or methionine type of binding.

Although we have advanced a hypothesis on the mechanism of Gram-stain reversal[7], we hardly know all the

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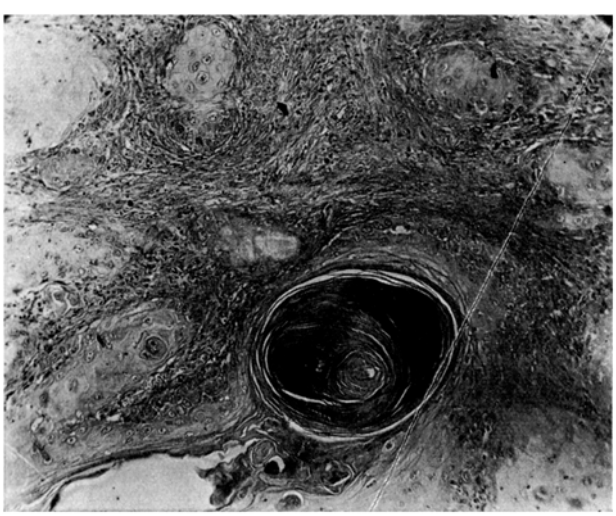
Some characteristic features of keratins

		α-pattern	Basophilia	Gram-staining response	Gram-staining behavior according to red.-ox.-scheme	SH- and other reducing groups	Remarks
Hard keratins (eukeratins) e.g. wool, hair, nails	Just formed	Quite stable in boiling water	–	–	Reversible	–	Circa has 12·5% of cystine
	Degraded	Still stable even after 22 h of degradation with 0·15 N sodium carbonate at 80°C	+ + +	+ + +	Reversible up to the 4 h degradation step	+ +	Circa 50% loss of cystine after 4 h of degradation
Soft (or pseudo-) keratins e.g. in epidermal and nervous tissue	Strat. germinat.	Unstable in hot water; changes to the β-pattern at 65°C in the SH-zone, or at 85°C in the S–S-layer respectively.	+ +	–		+ + +	
	Strat. spinosum		+ +	–		+ +	
	Strat. granulosum (keratohyalin)		+ + +	+	Irreversible	+ +	
	Strat. lucidum		–	+ +		+ +	Lower content of cystine and arginine and higher content of methionine as wool, hair and nails
	Strat. corneum		–	+ + +		–	

factors governing the Gram-staining response of proteins generally. There seems to be a concordance between the increasing intensity of Gram-positiveness (without counterstain) of wool, egg albumin, caseine, and epidermal keratin on the one hand and their increasing total amount of tyrosine histidine, arginine and lysine on the other.



Keratinizing squamous cell carcinoma of skin; Gram-stained without counterstain.

The selective and irreversible Gram-positiveness of keratinous structures in the epithelium is a constant feature and even in malignant tumors of stratified squamous epithelium in which the stratification is disorganized, keratin, wherever it occurs, stains Gram-positively (Figure). The Gram-staining (25 min with 30 s of "mordanting" with 1% of an alcoholic [80%] iodine) with no counterstain[1] reveals a characteristic keratin pearl which stains Gram-positively. When counterstain is used, e.g. a 0·5% solution of Bismarckbrown, the preceding Gentian violet treatment can be limited to 7 min without altering the subsequent treatment with the "mordant". If the 25 min Gram-staining without counterstain is used as in the Figure, other acidic structures such as nuclei also take the Gram-stain but can be prevented from doing so by treatment with nucleases prior to the Gram-staining procedure[1]; such a pretreatment does not affect the selectivity of the Gram-stain[2] for keratinous structures.

We believe that there might be many other possible uses of the described selectivity of the Gram-stain, and especially of the selective Gram-positiveness of soft keratins; e.g. to identify structures which may be keratinous, to stain the horny cells of the vaginal epithelium or for other diagnostic procedures, e.g. in the detection of bronchial carcinoma revealed through the presence of keratinous cell residues in the sputum or bronchial washing fluid, etc.

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Zusammenfassung

Die Selektivität der Gram-Färbung für bestimmte Keratine wird postuliert. Das Gram-negative Verhalten harter Keratine (zum Beispiel Wolle) kann durch alkalische und reduzierende Behandlung zum Gram-positiven färberischen Verhalten konvertiert werden; saure und oxydierende Agentien können die so erzielte Konversion wiederum rückgängig machen. Das Gram-positive färberische Verhalten weicher Keratine hingegen (zum Beispiel des Epitheliums) ist unter den Bedingungen des obigen Konversionsschemas irreversibel.

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