Experiment	**Mechanism of action of Bactrim, one of the best known antiinfectives**
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Reading	Chapters 18.6, 18.7, and "Monomers of nucleic acids: Nucleotides" in 4.15 in BBOM (9th ed.): Madigan M.T., J.M. Martinko and J. Parker: "Brock - Biology of Microorganisms", 9th Edition, Prentice Hall, 1999. ISBN: 0-13-085264-3
Objectives	To learn something about the story, that comes before resistance, i.e. the lethal target and the mechanisms of action of one exemplifying antimicrobial drug.
Background	**The significance of antimicrobial resistance** The introduction of antiinfectives is considered one of the most important achievements in the medicine of the 20th century. Antiinfectives are classified into 2 groups, (i) synthetic antimicrobial chemotherapeutics and (ii) antibiotics (products of certain microorganisms that are toxic to other microorganisms, such as human pathogens). Examples for (i) are sulfonamides and trimethoprim, examples for (ii) are penicillins, cephalosporins, tetracyclines, aminoglycosides, macrolides, quinolones and others. Antiinfectives allowed to save millions of lives and to cure even more. They allowed to break fear of the great bacterial diseases that had plagued mankind for centuries. Despite this immense success, bacterial infections have not disappeared. On the contrary: in certain geographical areas as well as in certain environments they keep becoming more frequent and more dangerous, even though the spectrum of pathogens has changed dramatically from *Yersinia pestis*, *Vibrio cholerae* and *Salmonella typhi* to opportunistic pathogens such as *Escherichia coli*, staphylococci, and *Pseudomonas aeruginosa*. Why is that so? The answer is resistance. Bacteria have learned to cope with antimicrobials through evolutionary adaptation. Moreover, parasexual mechanisms of gene transfer have enabled them to pass on highly effective resistance genes to species of even remote relationship. Integration of such genes into self-replicating genetic units, plasmids, and accumulation of several such genes on single plasmids have caused the appearance of multiply resistant strains of species otherwise fully susceptible. Such organisms thrive under selection pressure, and selection is highest where great quantities of antimicrobials are applied, which is in hospitals. The situation is such, that clinical isolates of patients suffering from infections have to be tested for susceptibility, in order to detect such acquired resistances. These resistance tests are the most important task of Medical Microbiology. The results, called antibiograms, are reported to the clinician and they are the basis for his decision on which antibiotic to use best. **Mechanisms of action and mechanisms of resistance** It is obviously important to investigate the mechanisms of resistance in order to take counter action. One example of such research is illustrated by Prof. Brigitte Berger-Bächi in her experiment no. 15. In order to study the mechanisms of resistance, it is a pre-condition to know something about the mechanisms of action of the involved antimicrobials. One such mechanism shall be illustrated by the presented experiment.

Mechanism of action of sulfonamides

Sulfonamides are fully synthetic compounds not found anywhere in Nature. They were derived from azo-stains by a German scientist, Gerhard Domagk in 1934. Some of them are still in use today, although they are now applied as combination formulations, together with trimethoprim, in order to lower the rate of resistance. The most popular such formulation is co-trimoxazole, better known under the trade name Bactrim.

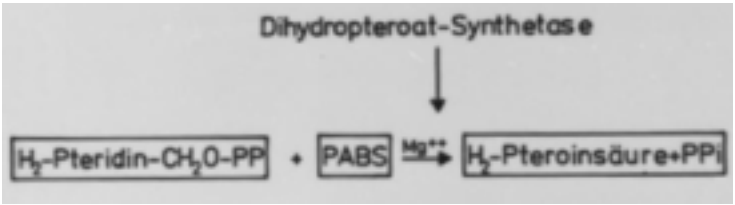
Sulfonamides interfere with the biosynthetic pathway of tetra-hydro-folic acid (THF) such that THF is no longer produced. This causes lethal deprivement of nucleic acids, because THF is an essential co-factor in the metabolism of nucleic acids.

Why are sulfonamides so selectively toxic to bacteria but not to host cells? The reason is very simple: bacteria have to synthesize their own THF. Human cells are unable to synthesize THF, because the genetic tools for this pathway are lacking. As a consequence, humans (and animals) have to eat THF as a vitamin, so sulfonamides cannot interfere with their metabolism.

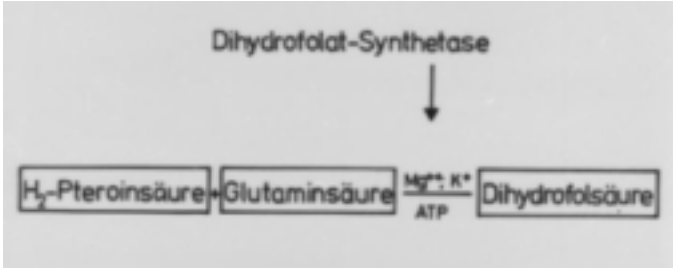
Detailed mechanism

The biosynthetic pathway shows that THF is built together from 3 precursor molecules: Di-hydro-pterin (H2pterin), para-amino-benzoic acid (PABA), and glutamic acid. The reaction steps are as follows:

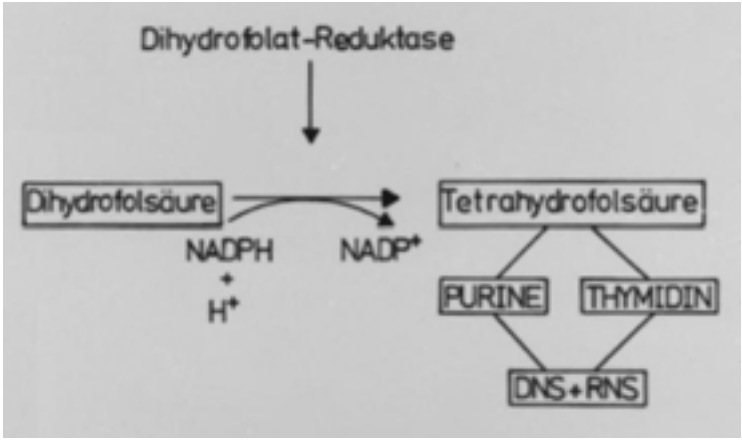
1) H2pterin and PABA are linked together by H2pteroate synthetase:



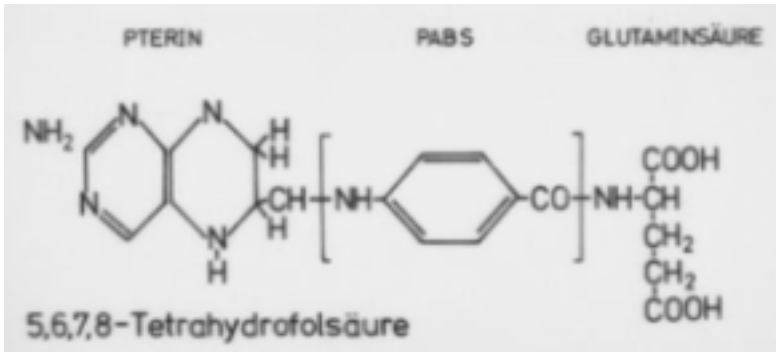
2) H2pteroate and glutamic acid are bonded by H2folate synthetase to form H2folic acid:



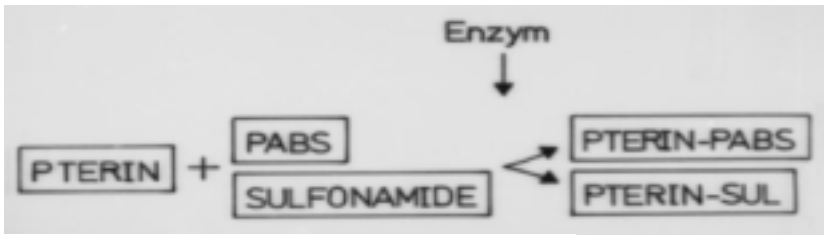
3) H2folic acid is finally reduced to THF by H2folate reductase:

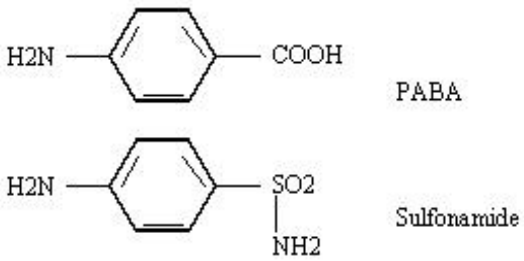


4) The structural formula of THF is as follows:



The sulfonamide is a structural analogon of PABA, and therefore competes with PABA for binding to the active site of H2peroate synthetase. As a consequence, instead of the functional H2pterin/PABA (i.e. H2pteroate) a H2pterin/sulfonamide complex is formed which is biologically inactive and does not lead to THF:





Literature

http://www.vet.purdue.edu/depts/bms/courses/bms514/chmrx/sulfashd.htm

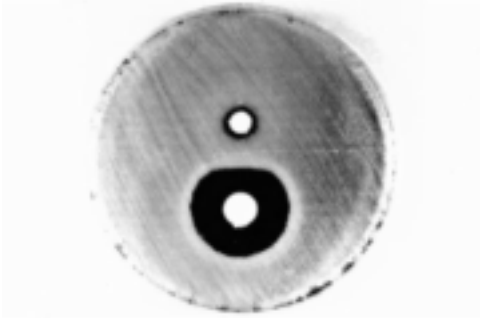
www. Links

see above

Practical work

Presented experiment

You shall perform an experiment to show the competitive inhibition of H2pteroate synthetase (seen as a growth inhibition), as well as the reversal of this inhibition by an excess of PABA:



Material and Experimental Protocols	**Materials** Bacteria: - *Eschrichia coli* B (41): susceptible to sulfonamides (on sheep blood agar) Other materials: - Müller-Hinton Agar plate - Sterile 0.9% NaCl (physiological concentration) - Swabs - Turbidity standard McFarland 0.5 ==> Equivalent to 10^8 bacteria/ml - Paper discs impregnated with sulfonamide - Paper discs impregnated with PABA - sterile Squeezers **Protocol** 1. Pick 3-5 colonies of *E. coli* B from the plate with a swab and suspend in NaCl. 2. Compare suspension with turbidity standard and dilute, until the turbidity is equal. 3. Soak a fresh swab within the bacterial suspension, and inoculate one Müller-Hinton plate evenly with it. 4. Using the squeezers, apply one disc with sulfonamide and one with PABA, and place them approximately 10 to 15 mm next to each other. 5. Incubate o/n at 35°C 6. Examine and interpret the shape of the inhibition zone around the sulfonamide disc.
Laboratory Rules & Precautions	*E. coli* is a normal inhabitant of the human and animal intestine. It belongs to risk group 1 and does not pose a health risk. Nevertheless, care must be taken not to contaminate oneself, fellow students or the environment. Aerosol formation has to be prevented. All waste must be sterilized before disposal. Hands must be washed and desinfected and surfaces wiped with 70 % ethanol after completion of the experiments.
Experiences gained	• Aseptic handling of bacterial pure cultures • Disc susceptibility testing according to Kirby Bauer, that is still widely used in the clinical microbiology laboratory • Insight into mechanistic pathways of antiinfectives
Timing	15 min (experiment can be performed e.g. while tissue is fixed or stained with experiment no. 14)
Reporting	○ Draw the result ○ Explain the antagonistic effect that you find
Questions to be answered	○ What is the principal difference between the prokaryotic and eukaryotic metabolism with respect to the sulfonamides, and what are the consequences of it? ○ Can you think of resistance mechanisms to evade the effects of sulfonamides? ○ What is the mechanism of action of trimethoprim, and why does it act synergistically in combination with sulfonamides? (Synergy means: the inhibitory effects are not simply additive, but the two compounds potentiate each others effects). To be answered after study of the cited literature.