

Discussion Of The Use Of RNAi As A Potential Cure For Equine Hyperkalemic Periodic Paralysis

By
Jordan Sinclair



Figure 1

PASS WITH DISTINCTION

Research Paper
Based On
Pathology Lectures
At Vet-Medlink 2009

Abstract

Having researched the mechanism of RNAi, I decided to research equine genetic diseases for which RNAi may be able to cure or treat. Hyperkalemic Periodic Paralysis (HYPP) is the only dominant genetic disease found in horses. While HYPP also occurs in humans, I decided to focus on the equine strain due to researching the uses of RNAi from a veterinary point of view. It is a potentially lethal condition found primarily in American Quarter horses, of which the cause lies within a mutated gene that could well be silenced using RNAi. I found that, while RNAi theoretically would offer a cure (something currently unavailable) for this disease, it is unlikely that it would be taken up by many breeders due to the muscular physique (Figure 1) also caused by the same gene defect.

Introduction

In order to discuss the possible use of RNAi (RNA interference) as treatment for equine HYPP, we first must understand the process of RNAi itself. To do this, we must also understand the similarities and differences between the structure of RNA (Ribose Nucleic Acid) and DNA (Deoxyribose Nucleic Acid) and understand the process of protein production with cells.

DNA is made up of monomer units called nucleotides. Each nucleotide consists of 3 parts: a pentose sugar (deoxyribose), a phosphate group and a base. The DNA nucleotides differ from each other in the bases, as there are four possibilities: Adenine (A), thymine (T), guanine (G) and cytosine (C). The nucleotides join by covalent bonds between the sugar and phosphate molecules to form a polynucleotide chain with a sugar-phosphate backbone. Two of these chains run antiparallel to each other and hydrogen bonds form between bases on opposite chains, according to the base-pairing rules:

- A-T
- C-G

These chains twist into a double helix shape, which is the DNA molecule.

RNA is also made up of nucleotide monomer units. However, RNA nucleotides contain Ribose as the pentose sugar, the same phosphate group, and one of four bases. These bases are A, C, G and uracil (U). Therefore, RNA has slightly different base-pairing rules:

- A-U
- C-G

RNA is, however, a single stranded polynucleotide chain, and so the base pairing rules are important only during the process of protein production.

There are different types of RNA. The 3 main ones involved in protein synthesis within cells are mRNA (messenger RNA), rRNA (ribosomal RNA, which makes up the ribosome) and tRNA (transfer RNA). [1]

Protein synthesis can be split into two main stages: Transcription and translation.

1. Transcription is the copying of the instructions from the gene (a length of DNA coding for a particular polypeptide/protein molecule) in the nucleus. Here, the hydrogen bonds between the two polynucleotide chains in DNA split, and the helix partly unravels (not entirely), exposing the gene that needs to be copied. The similarity of the RNA and DNA base pairing rules allows free RNA nucleotides to hydrogen bond to the bases on the exposed DNA nucleotides. The DNA strand that the RNA nucleotides bond with is the 'template strand.' RNA polymerase enzymes catalyse the formation of the covalent bonds that secure the sugar-phosphate backbone of the new mRNA polynucleotide chain. This new mRNA molecule is, therefore a copy of the other DNA strand that had

partially 'unzipped,' which is called the coding strand. The mRNA molecule then peels away from the DNA and leaves the nucleus via a nuclear pore.

2. **Translation** is the production of the protein itself, which occurs at the ribosome, when the mRNA arrives. tRNA molecules in the cytoplasm of the cell carry specific amino acids, which are attached by aminoacyl-tRNA synthetase enzymes.
3. The tRNA molecules have a triplet of bases (called anti-codons), dependent on the amino acid they carry. This triplet is complementary to the triplet on the mRNA molecule (codon). In this way, the correct tRNA molecules, bearing their specific amino acids, arrive at the ribosome in the correct order to synthesise the correct protein.
4. At the ribosome, peptide bonds form between the amino acids brought by the tRNA molecules to form a polypeptide (protein) chain. The ribosome moves along the mRNA molecule, one codon triplet at a time, each time adding another amino acid to the growing protein.
5. When the amino acid carried by the tRNA molecule forms a peptide bond with the polypeptide chain, the tRNA molecule is released from the ribosome. [1, 2]

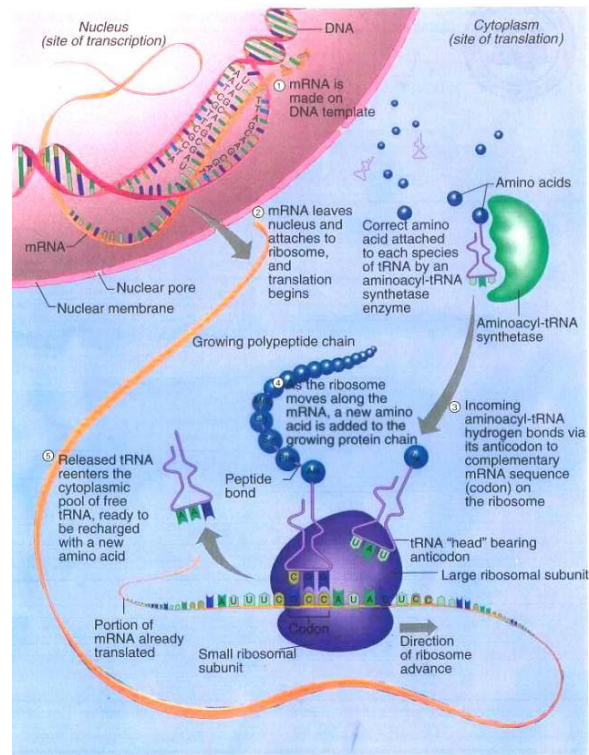


Figure 2: Protein Synthesis

The RNAi pathway prevents the mRNA molecule reaching the ribosome after transcription; translation never occurs. It was discovered accidentally during attempted genetic engineering of petunias around 1990. The intention was to add a gene to induce the red pigment to achieve a better colour of petal. However, the result was an effect called co-suppression, which in fact turned the petals white. This was not explained until Andrew Fire and Craig Mello's work on investigating genes in nematodes. [3]

The base sequence (the genetic code) in an mRNA molecule can be referred to as the 'sense' sequence. Therefore, the RNA molecule with the complementary sequence can be referred to as the 'anti-sense' RNA. Fire and Mello found that injecting particular sense and anti-sense RNA together into worms triggered twitching movements, similar to those seen in worms that lack a correctly working gene that codes for a specific muscle protein. It was explained that the sense and anti-sense RNA strands bind to form double-stranded RNA, dsRNA, which is the beginning of the RNAi mechanism. [3]

The mechanism of RNAi

- Long dsRNA (more than 200 nucleotides in length) is introduced to the cell.
- An RNAase III enzyme called 'dicer' cleaves the dsRNA strands into smaller fragments, now known as 'small interfering' RNA, siRNA. These are about 20 nucleotides in length.
- A protein complex called RISC (RNA-induced silencing complex), made mostly of the protein argonaute, binds to the siRNA. It eliminates the 'sense' strand of the siRNA.
- The anti-sense strand, which remains bound to RISC, aids it in detection of the mRNA molecule, to which it can bind by base pairing. Therefore, only the specific mRNA molecule will be destroyed.

- When RISC binds to the mRNA molecule, it cleaves it into fragments and therefore destroys it.
- Because the mRNA didn't reach the ribosome, translation didn't occur and so the gene that codes for the synthesis of that protein is silenced. [3,4, 5,6]

In mammals, introduction of long dsRNA triggers an inflammatory antiviral response, which shuts down all protein production, not just synthesis of the one coded for by the gene that is to be silenced. This is, however, resolved by introducing siRNA immediately, rather than dicer cleaving the dsRNA. [5, 6] To see the mechanism of RNAi in video form, see

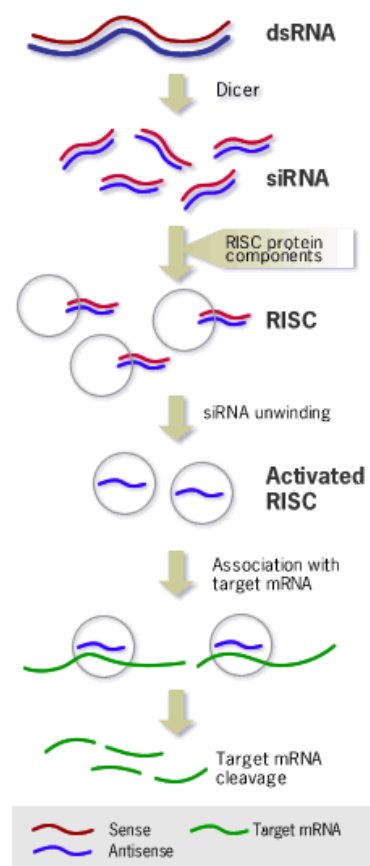


Figure 3:
Mechanism of RNAi

http://www.nature.com/focus/rnai/animations/rnai_revised_500x280.mov.

The question is, how does RNAi relate to the veterinary world? The possible uses of RNAi being used as post-transcriptional gene silencing include treatment for viruses, cancer and genetic disorders, such as equine HYPP, as I will discuss. siRNA has already been used to silence a gene causing high blood cholesterol levels in animals. [3]

Discussion

HYPP is a potentially lethal inherited genetic disease present in humans and horses, in which a genetic mutation alters the functioning of the sodium channels in muscle cells [12]. The name suggests that excessive potassium leads to repeated episodes of paralysis, however, while increased potassium can trigger episodes, it is not the initial cause of the condition [10, 11].

The genetic disease in horses has been traced back to a particular Quarterhorse stallion, "Impressive", who was known for his desirable musculature which allowed him to become a champion in many halter classes in America. As the defective gene is autosomal and dominant, every horse in the "Impressive" bloodline would have at least one copy of the gene, and so would be at risk of HYPP episodes. Heterozygous horses (1 copy of defective gene) are less likely to develop symptoms than homozygous horses (2 copies of the defective gene). However, both could show signs of the disease at any point. Unfortunately, Impressive's desired musculature resulted in him becoming a leading sire, and so many Quarterhorse-type horses (including appaloosas, paint horses and palominos) inherited the disease. And so HYPP was soon a huge issue in America. [12, 13]

The gene responsible for the defect is the SCN4A gene, otherwise referred to as the 'alpha subunit of type IV voltage-gated sodium channel.' This gene provides the instructions for making the sodium channel proteins present in muscle cell membranes [9]. Sodium is usually maintained in high levels in the blood (outside of cells) and potassium is kept at high levels inside the cells. These are known as electrolytes, and their balance allows electrical signals to be

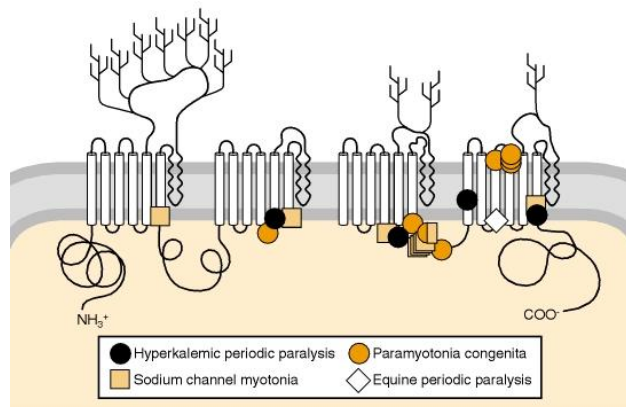


Figure 4: Approximate Location Of The HYPP Gene In The Sodium Channel

generated and transmitted to trigger muscle contractions for the body's normal functioning. Normal horses are generally unaffected by small changes in potassium levels in the bloodstream [12].

In HYPP horses, a mutation in the SCN4A gene (a guanine molecule is present in HYPP horses where a cytosine molecule would be present in normal horses) causes a disruption in the amino acid sequence (primary structure) of the sodium channel protein: a leucine amino acid is added instead of a phenylalanine during translation. Because the leucine molecule is smaller, physical or electrochemical leaking of sodium occurs when the channel should be closed [10]. Therefore, higher potassium levels in the blood of HYPP horses upsets the balance, causing more sodium to leak through the channel when it would be closed, resulting in sustained high levels of sodium inside muscle cells. It is this that causes the symptoms of HYPP.

Symptoms of attacks include mild muscle twitching, hind quarter paralysis, excessive yawning, prolapse of the third eyelid or abnormal whinnying (voicebox muscles are also affected). Ultimately, the muscles surrounding the heart and lungs could become paralysed, causing heart attack or suffocation and therefore death [11, 12].

Currently, owners are advised to 'manage' the problem once a horse is diagnosed with HYPP. The only reliable diagnostic technique is a genetic test performed on a blood sample. The main way to prevent HYPP attacks is by restricting the potassium intake in the diet. This can be done by feeding smaller, more regular (as many as possible) concentrate feeds that are themselves low in potassium such as oats, barley and sugarbeet. Molasses and alfalfa should be avoided, since they contain high potassium levels. With regards to forage, most hays have high potassium content. Therefore, turnout is recommended as the high water content of grass will keep ingested potassium levels low. This is also beneficial to the horse as regular exercise is also recommended for HYPP horses [10, 12, 13]. Another method of managing HYPP is by using acetazolamide, a drug that lowers potassium levels by increasing urination. While some owners use this daily, it is also used during a moderate to severe attack to encourage excretion of potassium. Other treatments during the attack itself include intravenous administration of dextrose or calcium. The sugar, dextrose, will cause the body to release insulin, which will drive potassium back into cells (out of the bloodstream). Insulin may also be given directly. Calcium will counter the effects of potassium [12, 13].

Recent research into "HYPP Solution" resulted in the development of a liquid intended to immobilize HYPP positive sperm cells when mixed with semen so only negative sperm cells (those not carrying the gene) would be left to inseminate. This technique would have been used to stop the HYPP gene defect being inherited from the sire to the foal. However, the result was that 50% of the sperm cells were immobilized, but these were a

mixture of HYPP positive and negative cells, therefore leaving a mixture to inseminate [11].

Another study involved observing the effects of local anesthetics on rat skeletal muscle cell sodium channels containing the equine HYPP gene defect. This resulted in the conclusion that anesthetics did not have the capacity to improve functioning of sodium channels affected by the mutation [15].

At the moment, it is clear that no cures as such are currently available for horses with HYPP. The disease is inherited and symptoms can be prevented or managed but not eradicated entirely. However, I think the attempts at tackling the disease have thus far involved external factors only and altering the disease at a cellular level has not been considered. Here, my suggestion for the use of RNAi becomes evident.

RNAi and HYPP

Since RNAi has the ability to silence defective genes, could the mutation in the SCN4A gene not too be silenced with this method? We know the substitution of a guanine base for a cytosine base in the gene itself is the mutation causing the wrong amino acid to be added in the polypeptide chain. We also know that RNAi can silence genes coding for a specific protein by preventing translation. The method of RNAi would have to involve direct introduction of siRNAs as we know that the dsRNAs which are cleaved into siRNAs can trigger the inflammatory response in mammals. [5] Therefore, siRNA molecules with anti-sense strands that are complementary to the mRNA that carries the copy of the SCN4A gene would need to be produced and transferred into the cell. This siRNA would bind to RISC and the sense strand would be eliminated. The anti-sense strand would therefore be able to bind to the SCN4A mRNA molecule by complementary base-pairing, which allows RISC to cleave the mRNA into fragments. The mRNA molecule would never reach the ribosome, preventing translation and so synthesis of the defected sodium channel responsible for HYPP.

Since dsRNAs can't be used due to the inflammatory response they would trigger, we need to consider delivery methods of siRNA. [6] Transfection is the term used for deliberate introduction of genetic material into cells. Transfection can be 'transient' (short term) or 'stable' (long term). There are many different types of transient transfection, using different methods. [16] Most of these are problematic as they are inefficient, can be toxic to cells and are often inconvenient. Electroporation particularly causes high rates of cell death. The most efficient method of transient transfection of siRNA is a type of chemical-based transfection involving the use of cationic lipids. Cationic lipids have a positively charged head group with one or more hydrocarbon tails. This positive charge allows interaction with the siRNA molecule, which, when attached to the lipid molecule can be taken into the cell by endocytosis. This method is efficient, relatively simple, works for a wide variety of cells and can be consistently reproduced. [19, 20, 22]

The RNAi mechanism triggered by this method of siRNA delivery can function for several days and so the silencing effect on the gene also lasts as long. Although it does also appear to be transferred to daughter cells, the effect is not permanent and therefore the SCN4A gene would code for the faulty membrane channel protein once more, resulting in HYPP symptoms appearing again. Therefore, using any type of transient transfection to deliver siRNAs to the cells would mean continuous treatment, every few days, bringing the solution back to a method of managing the disease rather than curing it. [18]

Instead of transient transfection, expression vectors could be used. These are molecules that contain a copy of a small DNA insert (about 70 base pairs) that continuously release siRNAs or shRNAs (short/small hairpin RNAs) [21]. shRNAs contain a sense strand and an anti-sense strand, with a small loop containing a different sequence between the two strands. Because the sense and anti-sense sequences are complimentary, the molecule curls round in a 'hairpin' shape. On entering the cell, DICER cleaves the shRNA into the required siRNAs and so the RNAi mechanism can proceed. The vectors contain RNA polymerase III promoters, an enzyme that initiates synthesis of the shRNAs or separate sense and anti-sense strands of siRNAs, which recombine in the cell to form siRNA. [5, 18, 22].

We still need to consider methods of delivering the expression vectors themselves. This could be done using the transfection methods described or stable transfection such as viral vectors, which are highly efficient. [23] The viral vectors infect the cell with the corresponding virus, at the same time delivering siRNA or shRNA for RNAi. There are 3 viral delivery pathways; lentivirus, adenovirus (both of which infect dividing and non-dividing cells) and retrovirus (which infects dividing cells only). [22] Examples of viral vectors include HIV (Human Immunodeficiency Virus), which is a lentivirus, and EIAV (Equine Infectious Anemia Virus), which is a retrovirus. The issue with using viral vectors is the possibility of the virus self-replicating and entirely infecting the organism, leaving it with an additional disorder to the original disease.[24, 25, 26]

Ethical Issues

As with any kind of gene therapy, there are many ethical issues regarding RNAi. According to Beauchamp and Childress, there are the "4 principles of biomedical ethics" to consider. These principles are:

1. Respect for Autonomy (all involved parties must consent and it is accepted that the decision of others may be different); It is doubtful that all owners or breeders would consent to the use of RNAi to treat HYPP due to the muscular physique that would be lost as a result of doing so.
2. Beneficence ('good' is promoted and the benefits evaluated); RNAi offers a cure, opposed to another management technique for HYPP.
3. Non-maleficence (evil/harm is minimised or not inflicted and all risks assessed); If viral vectors were used for RNAi and the virus were to self-replicate, the horse could end up in a much worse condition than it was to begin with. The viral vectors could also trigger an immune response or cause permanent (unwanted) changes of DNA in the cell, which could result in cancer. [17, 25]
4. Justice (fairness and equality considered); the high cost of any gene therapy would apply to RNAi especially since it is complex and a very recent branch of gene technology, which may result in it not being fairly available to those owners who would be willing to use this method of treatment but simply cannot afford it.

[27,28]

There are also religious arguments present. The use of RNAi could be seen as 'playing God,' since it is questionable as to whether humans have the right to change the genetic makeup of an organism or the natural way a cell operates. However, there is also the argument that God gave us the intelligence to do so. [29, 30]

Conclusion

While it would appear that RNAi would be a suitable treatment for HYPP as the disease is caused by a genetic defect, there are still many issues to consider. The main benefit of the use of a genetic treatment such as RNAi for HYPP would be that it can potentially offer a cure, which is not currently available. However, it would appear that this could only be achievable by the use of viral or expression vectors, as these would continually release shRNAs, which can be cleaved into siRNAs by DICER for use in the RNAi mechanism. This continual release would suggest a 'one-off' treatment that would last for the duration of the affected horse's life. However, if transient transfection of siRNAs were used, the duration of the silencing effect would be limited, which makes it seem like a method of management opposed to a cure as such. Because there are many methods of management used currently that are fairly successful in at least prolonging the horse's life, I think the expense of the RNAi treatment would discourage owners to use it.

The RNAi would indeed appear to eliminate the affected SCN4A gene before it would translate in the ribosome and produce the leaky sodium channel in the membrane. However, if the gene coding for that sodium channel is silenced, then surely none of those channel proteins would be made and so none would be present in the membrane. If there are none of these particular channels, this could dramatically effect muscle function. Therefore, not only does the faulty gene need to be silenced, but there could also be the possibility that a replacement, 'correct' gene would need to be introduced in order to allow synthesis of the correctly functioning sodium channels.

Also, the injection sites would need to be considered as the siRNAs or vectors may need to be delivered to every muscle or every affected cell. Of course, it would be a very complex procedure to inject every muscle, some of which would be difficult to access. Therefore, the idea of using viral vectors, which, after administered via injection, should spread throughout the entire body appears to be the best option. If a lentivirus such as HIV were used, it would infect both dividing and non-dividing cells, appearing to do just this. However, the issue with using HIV as a viral vector would be the risk of self-replication, infecting the horse with HIV itself.

Although I feel that use of RNAi to treat HYPP would be ideal, since it offers a cure rather than just the management techniques that are currently used, I think the initial off-putting factor would be the high cost, which is expected of any genetic treatment. I also think that, because curing HYPP would eradicate the muscular physique it causes in horses, breeders would be unwilling to take up treatment. It has been previously suggested that breeding with HYPP horses could be banned to eradicate the disease. Since breeders would not conform to this or use any of the management techniques currently available, it seems unlikely that they would be willing to uptake an alternative method of eradication. [11] While this seems cruel, the greedy nature of such breeders and their desire only for the success of horses in the halter classes unfortunately blinds them to the possibility of a fantastic cure for such a horrific condition that can be lethal to these animals. Therefore, I think that, should RNAi become available as a permanent cure for HYPP, it may be taken up by some owners but not those breeders whose desire to make money from valuable prizewinning horses would overrule their humanity.

References

1. Kennedy, P and Sochacki, F. (2008) OCR Biology, Heinemann.
2. Oxlade, E. (2007) Genetics: The science of genetics revealed, Studymates.
3. Nobel prize text: http://nobelprize.org/nobel_prizes/medicine/laureates/2006/press.html
4. http://www.rnaiweb.com/RNAi/What_is_RNAi/index.html
5. http://www.rnaiweb.com/RNAi/RNAi_Glossary/
6. http://www.ambion.com/techlib/append/RNAi_mechanism.html
7. <http://cmbi.bjmu.edu.cn/news/report/2004/biotech/71.pdf>
8. <https://www.netpets.org/horses/healthspa/hypp.html>
9. <http://ghr.nlm.nih.gov/gene=scn4a>
10. <http://www.admani.com/AllianceEquine/TechBulletins/HYPP.htm>
11. <http://www.bringinglighttohypp.org/>
12. <http://www.tufts.edu/vet/sports/hypp.html#2>
13. http://www.petalia.com.au/Templates/StoryTemplate_Process.cfm?specie=Horses&story_no=1940#ct-4
14. <http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=gene&part=hyper-pp>
15. <http://ajpcell.physiology.org/cgi/content/abstract/275/2/C389>
16. <http://en.wikipedia.org/wiki/Transfection>
17. <http://www.ambion.com/techlib/tn/131/5.html>
18. http://www.ambion.com/techlib/hottopics/rnai/rnai_may2002_7.html
19. http://www.genlantis.com/commerce/ccp1138-1313-genesilencer-reagent-gsr_p49.htm

20. http://www.invitrogen.com/etc/medialib/en/filelibrary/pdf.Par.75261.File.dat/F063727_MechCationic.pdf
21. http://www.genscript.com/siRNA_technology.html
22. <http://www.sabiosciences.com/newsletter/rnai.html>
23. <http://www.libpubmedia.co.uk/RNAiJ-Issues/Issue-1/Chen.pdf>
24. <http://www.faqs.org/patents/app/20090017543>
25. <http://biology.kenyon.edu/slonc/gene-web/Lentiviral/Lentivi2.html>
26. <http://jvi.asm.org/cgi/content/abstract/73/4/2762>
27. <http://www.medsci.org/v05p0159.htm>
28. http://www.uq.edu.au/oppe/PDFS/Ethics_primer.pdf
29. <http://www.srtp.org.uk/genthpy1.htm#Issues>
30. <http://www.youtube.com/watch?v=xtfYjL3F-MU>

Pictures

Figure 1

<http://www.lotsofpinesfarm.com/stallions/bucky/buckyfrm1ds.jpg>

Figure 2

<http://www.harford.edu/faculty/eaugusti/images/Protein%20Synthesis.JPG>

Figure 3

<http://www.ambion.com/jp/figs/f00231.gif>

Figure 4

<http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=bnchm&part=A3003>