THE STABILITY OF BIOLOGICAL STANDARDS

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SYNOPSIS

The authors emphasize the importance of stability in biological standards and discuss the steps taken to ensure the stability of International Standards and the theoretical basis of the various stability tests used.

THEORETICAL

The primary function of a biological standard is to provide a stable point of comparison against which the activity of other batches of similar material may be calibrated. It follows, therefore, that an unstable standard is completely valueless. Unfortunately, no direct method of testing stability exists. Nevertheless, the stability of International Standards is seldom questioned, and the general belief that they are stable is based upon comparatively well-founded tenets. It is the purpose of this paper to describe the various indirect measures that can be applied to the stability of biological standards and to illustrate, with special reference to serum standards, the validity of our belief in the stability of the International Standards.

Storage of standards

In the first place, we can attempt to prevent deterioration of the standards by efficient storage. In general, deterioration must be assumed to be the result of a chemical change in the preparation and consequently, we can make it difficult for chemical changes to occur. This is done by removing, as far as possible, five of the common factors favouring chemical reactions, namely: heat, light, water, oxygen, and micro-organisms.

Heat. All the International Standards are at present stored at a temperature of $-10^\circ$C. At this temperature, all chemical reactions are greatly

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retarded. However, it may not always be possible to store standards at this temperature. Thus, if a fluid preparation is used as a standard, physical changes occurring during freezing may themselves cause deterioration. Such preparations have to be stored at approximately +2°C. On the other hand, attenuated virus vaccines may turn out to be unstable even at −10°C, and may therefore require storage at −70°C in a mixture of alcohol and dry ice.

*Light.* Light is the least important of the potentially damaging factors as far as International Standards are concerned and also is the most easily excluded. All the International Standards are stored in the dark and this presents no problem whatsoever.

*Water.* Water, even in traces, is essential for many chemical reactions and in its total absence few such reactions occur. Thus, if it were possible to store all standards in a completely anhydrous form, few questions of stability would arise. Unfortunately, there are formidable obstacles to this course.

In the first place, total desiccation is usually impossible. The strongest drying agents acting in very high vacua will remove most but not all water. The reaction is an exponential one and complete desiccation is only obtained in an absolute vacuum, which is unattainable. We may, therefore, dry for 3 days over phosphorus pentoxide at a pressure of 5 microns of mercury and achieve 99.9% desiccation; this may be quite sufficient to make the chance of chemical reaction and deterioration unlikely in the extreme. Even this may, however, be undesirable for an entirely different reason. When a user opens an ampoule of an International Standard, he will often wish to weigh out a portion of the contents with a high degree of accuracy. The provision of material so hygroscopic that it takes up water during this process, even when all possible precautions are taken, may thus nullify the function of the standard. Consequently, severe drying of standards is avoided when this results in very hygroscopic material.

We are therefore unable sometimes to do all we would like to do in removing water from standard materials. This does not mean, however, that we cannot go a fair distance in this direction. We can set our faces firmly against fluid standards; in many cases we can desiccate over P₂O₅ at very low pressure; and we can nearly always dry as far as is compatible with avoiding a product so hygroscopic that it is useless in practice.

*Oxygen.* Most of the International Standards are dispensed in sealed tubes or vials filled with a dry inert gas, usually nitrogen. This is a relatively simple procedure and removes, almost completely, oxygen, another essential participant in many chemical reactions.

*Micro-organisms.* Chemical reactions causing deterioration are, in the case of sera and vaccines, frequently the result of growth of micro-organisms in materials which, by their very nature, are relatively good media for the
growth of such organisms. Growth of micro-organisms is, of course, minimized by the exclusion of heat, light, water, and oxygen; but as a further precaution, standards are normally dispensed as sterile products under aseptic conditions and the containers are sealed so as to exclude micro-organisms. In a few cases, e.g., pertussis vaccine, a small quantity of a bacteriostatic is also added.

Thus, all the conditions of storage of biological standards are chosen so as to minimize the chances of deterioration as a result of chemical reactions, and this is really the basic reason for our belief in their stability. Such conditions of storage will certainly prevent any rapid deterioration; and it is assumed that deterioration takes place extremely slowly, if at all.

In any case, this is the most that can be done to ensure stability. All the further investigations described in this paper represent only checks, and often only very crude checks, that our assumption of stability is justified.

Tests of stability of biological standards

The stability of a preparation requiring bio-assay is estimated by comparing its potency before and after storage with that of the appropriate standard, which is assumed to be stable. Thus no such direct test of the stability of the standard itself is possible. Nevertheless, there are a number of ways, all of them based on certain more or less doubtful assumptions, which may be used to test the stability of a standard. These are:

(1) comparison of the standard after storage at relatively high temperatures with the same material stored at $-10^\circ$C— the accelerated degradation test;

(2) comparison of the standard before and after storage with a number of similar preparations kept in the same way— the parallel stability test;

(3) comparison of the activity before and after storage in terms of a response in a population of animals— the animal-response stability test.

Of these, the accelerated degradation test has been fairly widely used; the other tests are probably little known and we have given the names used above for the sake of convenience. All of them are worth examining with a view to determining just what assumptions are made in their application and just how far we can rely on them as an indication of the stability of biological standards.

(1) The accelerated degradation test

In this test, samples of the standard are stored for a period at different temperatures, including $-10^\circ$C. Thereafter, the potencies are compared on an initial assumption that there is no deterioration at $-10^\circ$C. The test may then be continued by extending the period of storage and repeating the comparison.
The basic assumption is made that the chemical reaction accounting for the deterioration is a "monomolecular" one. For reactions of the monomolecular type at temperature \( T \), the rate constant \( (k_T) \) relating the potency \( (a-x) \) after time \( (t) \) to the initial potency \( (a) \) is:

\[
k_T = \frac{1}{t} \log \left( \frac{a}{a-x} \right)
\]

Thus, the experimental values for \( t, a, \) and \( a-x \), allow of the estimation of \( k_T \) for each temperature of storage other than \(-10^\circ\C (k_{-10} \) is presumed to be zero).

The Arrhenius equation linearly relates the log of the velocity constant to the reciprocal of the absolute temperature, \( T \). Thus:

\[
\log k_T - \log k_{T_1} = \frac{1}{T} - \frac{1}{T_1} \quad \log k_{T_2} - \log k_{T_1} = \frac{1}{T_2} - \frac{1}{T_1}
\]

From this equation, an estimate of the velocity constant \( (k_{-10}) \) at \(-10^\circ\C \) can be made, and by interpolation in Equation (1) an estimate of the loss of potency on storage at \(-10^\circ\C \). A further cycle of calculations could then be made using this calculated estimate of \( k_{-10} \) instead of the original assumed value of zero; and the cycle could be repeated until a constant estimate was obtained.\(^a\) This would, of course, be unnecessary if the initial estimate of \( k_{-10} \) were so small as to be negligible. A further check may be carried out by the extension of the storage time to \( t_2 \). This will provide a separate estimate of \( k_{-10} \) and should reinforce the conclusion drawn from the first experiment.

This type of experiment is often very useful, but it is well to be aware of the assumptions underlying it:

1. the assumption that the reaction is a monomolecular one;
2. the assumption that the relationship between the log potency and the time of storage is linear at all temperatures—Equation (1). Otherwise, the extrapolation of the stability at \(-10^\circ\C \) over a short period to storage over, say 20 years, is invalid;
3. the assumption that Equation (2) is true of the reaction, i.e., that only one chemical reaction is involved. This is often not true. The material may have an aqueous phase that may freeze at temperatures below zero and thus alter the nature of the reaction. Or, at higher temperatures, the rate of an enzymic reaction may be limited by enzymic destruction, etc.

\(^a\) In one set of data obtained on an insulin preparation, \( k_{-10} \) was presumed zero, and \( k_{10} \) and \( k_{20} \) were calculated to be 0.020 and 0.209 respectively. \( k_{10} \) was then estimated as 0.003. A further series of 5 cycles of calculation gave \( k_{10} \) values of 0.0073, 0.0082, 0.0087, and 0.0089. It thus appeared that the exponent of this series of estimates was approximately 0.009, and using this value, the loss of potency of the preparation was estimated at 2.05% per annum, whereas the original estimate of \( k_{-10} (0.003) \) suggested a loss of potency of only 1.2% per annum. These calculations are quoted by kind permission of Mr. P. A. Young of the Wellcome Research Laboratories, Beckenham, England.
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A more basic theoretical presentation of the effect of temperature on the rate of thermal inactivation is the following.

Thermal inactivation is assumed to be due to a change of specific atomic configurations, leading to a loss of biological activity. This breaking up of specific configurations is the effect of the vibration of many bonds by thermal agitation. The excitation is increased at high temperatures, and may cause the breakage of essential bonds involving an irreversible modification of the structure in a large fraction of the molecules, and a consequent loss of biological activity. For a configurational change to occur, a large molecule must attain a sufficient energy level. The total energy required at this activated level is called the heat of activation, \( \Delta H^\ddagger \).

This is balanced, to a certain extent, by an entropy term, \( T \Delta S^\ddagger \), expressing the increase of entropy due to an increase in the number of available vibrations by the opening up of the molecular structure and the release of bound water. \( \Delta S^\ddagger \) is called the entropy of activation.

The number of molecules reaching the required activated state (which is followed by a breakdown of the specific activity) will depend only on the temperature, the time, and the number of intact molecules present. Therefore, the fraction \( f \) of molecules remaining intact \( t \) seconds after exposure to a certain temperature is given by the following relation:

\[
f = e^{-Kt}
\]  

(3)

in which \( K \) is the reaction constant. This means, for instance, that 37% of the molecules will have remained unchanged after \( 1/K \) seconds. By measuring the fraction of activity remaining after a certain time of exposure to a certain temperature, \( K \) can be experimentally estimated. According to the theory of absolute reaction rates, the value of \( K \) is determined by the free energy of activation \( F^\ddagger \) and the absolute temperature \( T \), as follows:

\[
K = \frac{(kT/h) e^{-\Delta F^\ddagger/RT}}{e^{-\Delta S^\ddagger/RT}}
\]

where \( k \) is Boltzmann’s constant, \( h \) is Planck’s constant, and \( R \) the gas constant.

The substitution \( \Delta F^\ddagger = \Delta H^\ddagger - T \Delta S^\ddagger \) into this formula leads to:

\[
K = \frac{(kT/h) e^{-\Delta H^\ddagger/RT} e^{\Delta S^\ddagger/R}}{e^{\Delta S^\ddagger/RT}}
\]

or \( \log K = 10.3 + \log T - \frac{0.22 \Delta H^\ddagger}{T} + 0.22 \Delta S^\ddagger \)  

(4)

Having measured \( K \) experimentally at two different temperatures, we can calculate \( \Delta H^\ddagger \) and \( \Delta S^\ddagger \), and knowing these empirical constants we can then predict the rate of deterioration at any other temperature. Formula (4) will give us \( \Delta H \) in calories per mole, and \( \Delta S \) in calories per mole per degree.
It should be noted that this theoretical approach is also based on certain assumptions which are not necessarily true for each case, and that consequently the interpretation of the results must be made with caution and only in the light of our practical knowledge of the materials studied.

An experimental check of the validity of all these assumptions would be an impossible task, involving an enormous number of bio-assays. Moreover, it is extremely improbable in some cases, e.g., sera, that all the assumptions are valid. Thus, in spite of the useful information that such tests yield, they must be interpreted with caution.

(2) The parallel stability test

This is a type of test which is seldom planned but which, in the case of International Standards is often carried out fortuitously. If two similar preparations are made and stored under similar conditions, and repeated comparisons after different periods of storage show that the potency ratio of the two preparations is a constant, then two conclusions are possible—first, that both are stable, and secondly, that both deteriorate in parallel at the same rate. If this type of test is extended to three preparations, such parallelism is even more striking, and the second conclusion becomes more unlikely the larger the number of preparations studied. It would be quite possible to design a test of this kind, but in practice the use of the International Standards to calibrate National Standards serves just this purpose. If a number of National Standards are made and are frequently compared with the International Standard, and if the potency ratios remain constant, it is strong presumptive evidence that all the preparations are stable.

Nevertheless, it is only presumptive evidence. The possibility always exists that these similar preparations are all subject to the same particular type of chemical degeneration proceeding at the same rate, but this possibility is the less likely the less pure the preparations are. Thus, while several highly purified preparations, e.g., insulin, might conceivably deteriorate at the same rate due to one particular chemical change, several very impure preparations, perhaps varying widely from each other, are unlikely to do so. This is especially true of such preparations as antitoxic sera and vaccines. Moreover, there are two further observations to be made: first, that the error of bio-assay is often so large that the limitations of time and money make it impracticable to carry out comparisons which are sufficiently accurate to allow of the detection of minor degrees of deterioration, and secondly, that not all International Standards are for substances for which a number of such National Standards exist. This last point emphasizes the desirability of the continuation and extension of the present practice of creating, wherever possible, National as well as International Standards; the use of a large batch of material to provide for both International and a
number of National Standards, as is planned for the Fourth International Standard for Insulin, is only justifiable when the preparation is relatively pure (see above) and when a very large amount of evidence exists of the stability of the particular substance concerned.

(3) The animal-response stability test

In the early days of bio-assay, before the introduction of biological standards, the activity of most substances requiring bio-assay was expressed in "animal units". The undesirability of this practice has often been pointed out but it has not yet completely disappeared. This undesirability is based on the fact that the response of a group of animals is dependent on the design of the test, on the environmental conditions, and on the strains or stocks of animals used. All these factors may vary from day to day, and reliable and reproducible estimates of potency in "animal units" are seldom attainable. Nevertheless, within any one inbred colony of animals, kept for years under uniform conditions, the dose of one particular preparation—the standard—needed to produce, in one particular type of assay, a fixed animal response may be fairly constant. It may, in any case, be sufficiently constant to enable a continuous trend in any one direction to be apparent. If this trend is such that larger and larger doses of the standard become necessary to produce a fixed response, two conclusions are possible, first, that the standard is deteriorating, and, secondly, that the animal colony is becoming less sensitive or more resistant. Presumptive evidence against the second conclusion may exist in the form that numerous commercial batches of the substance, assayed always in comparison with the standard, show no such trend over the same period.

The limitations of this type of test are obvious. As indicated, it is usually not a "planned" test but is the outcome of years of control assays, none of which is highly accurate in the statistical sense, but all of which, taken together, may show a highly significant trend. It is seldom that such evidence can be used to provide a qualitative estimate of the degree of deterioration. The most that can be expected is a suggestion that all is not well with the standard preparation.

In addition to these three methods there is another, relatively rare, opportunity for checking, in retrospect, the stability of a standard. This occurs when stocks of an existing standard run out and a new standard is made to replace it. Thus, for example, an International Standard for Penicillin was established in 1944, and was replaced in 1952. The difference in potency between the old and the new standards was found, in an extremely extensive and accurate bio-assay (which can only be achieved on such an occasion when continuity of the international unit is essential) to be only 1.5%. Thus, assuming that manufacturing methods did not, in 1952, yield a less pure preparation than they did in 1944, the maximum loss of potency of the old standard over the eight years of its life was 1.5%.

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EXPERIMENTAL

Studies of the Stability of International Serum Standards

In 1951, the Expert Committee on Biological Standardization of the World Health Organization decided to collect the existing evidence of stability of the serum standards, and also asked the Statens Seruminstitut to test stability by subjecting standard preparations to degradation tests at different temperatures for varying periods of time, in the hope of estimating the probable rate of deterioration of standards held both in cold storage and at laboratory temperatures. Such tests have now been carried out, and the following is a survey of this work.

From a practical point of view, we should like to be able to answer two questions: (1) What is the stability of the international serum standards, kept under constant cold storage in the dry state? (2) What is the stability of the solutions of these standards as dispensed and distributed to other laboratories? To answer the first question we must consider periods of many years at temperatures of $-10^\circ C$ to $-15^\circ C$. The answer to the second question must deal with periods of weeks or months at temperatures that may occur during transport and under laboratory conditions.

Stability tests of dried standards

(a) Animal-response stability test. In 1928, we measured the amount of the International Standard for Diphtheria Antitoxin required to neutralize a certain quantity of a preparation of diphtheria toxin called 43/28. Repeated measurements have since been made every year, and during 25 years the results have not shown a significant change. During the same time, no decrease of the toxicity of the toxin preparation 43/28 could be observed by repeated measurements of the amount of this toxin required to kill a guinea-pig, or to produce a skin response in rabbits. We may therefore conclude that the International Standard for Diphtheria Antitoxin has suffered no significant deterioration.

The weight of this conclusion, however, is somewhat limited. Even though the toxin preparation 43/28 shows no sign of increasing $LD_{50}$ towards guinea-pigs, some deterioration of toxicity may have taken place, since, say a 10% shift would not be readily detectable. Also, the guinea-pig susceptibility may have changed during all these years. Therefore, the deterioration of a similar fraction of the International Standard Preparation of Diphtheria Antitoxin could escape our notice.

(b) Parallel stability test. The observed constancy of the relative potencies of other old preparations of diphtheria antitoxin as measured against
the International Standard suffers from a similar limitation, since all these preparations may conceivably have deteriorated at the same rate. Common sense, however, makes us feel confident that the loss of antitoxicity of a given weight of the International Standard for Diphtheria Antitoxin during the past 25 years of cold storage is extremely small, certainly less than 10% and probably negligible.

(c) Accelerated degradation test. In order to make deterioration proceed at a measurable rate, samples of the dry material in the original stock ampoules were subjected to higher temperatures. This was done with the International Standard Preparations of Tetanus Antitoxin, Antidysentery Serum (Shiga), Diphtheria Antitoxin, and Gas-Gangrene Antitoxin (Histolyticus). Samples of the two first mentioned were held at 37°C and at 56°C for eight weeks, and samples of the three last mentioned were kept for 5 months at 18°C, 37°C, and 56°C. No deterioration of antibody activity could be detected after storage for these periods at 18°C or 37°C. At 56°C the antidysentery serum lost about 16% of its original potency during 8 weeks and nearly 50% in five months. The tetanus antitoxin lost about 14% in 8 weeks at 56°C. The diphtheria antitoxin lost about 20% during 5 months at 56°C whereas no loss in potency of the gas-gangrene antitoxin (histolyticus) could be demonstrated after the same treatment.

These results showed that among the standard sera tried, the International Standard of Antidysentery Serum (Shiga) was the least stable at 56°C. If we admit that a 10% deterioration of this material during 5 months at 37°C may have escaped our notice, the rate of deterioration at 56°C would still be more than six times higher than at 37°C. By these assumptions, and by furthermore assuming $Q_{10}$ to be a constant in this range, the rate of deterioration at a temperature 70°C lower ($-14°C$) would at least be 640 times slower that at 56°C. This would mean, since the half-life at 56°C was approximately 5 months, that the half-life of the International Standard Preparation of Antidysentery Serum (Shiga) at $-14°C$ is at least 640 x 5 months, or 260 years. A half-life of 260 years is the same as a 10% loss of potency in 40 years.

Among the assumptions underlying this calculation is a 10% deterioration during 5 months at 37°C, whereas in fact we did not observe any deterioration. By admitting this possibility on account of the practical limitations of the measurement of potency of antidysentery serum, we have on purpose biased the result towards as high a rate of deterioration at $-14°C$ as is reasonably conceivable. Probably, therefore, the International Standard for Antidysentery Serum will suffer a smaller loss than 10% during 40 years of cold storage. Furthermore, since a 10% difference in potency is within the limits of error of any practical potency measurement likely to be carried out with the help of this International Standard, it would be hard to point to any practical consequence of a barely possible 10%
deterioration during 40 years. The International Standard for Anti-dysentery Serum was established in 1928, so there would seem to be no cause for doubting the continued usefulness of this standard for the reason of instability.

The same conclusion applies to the other international serum standards investigated, since all were more stable than the antidysentery serum standard when subjected to 56°C. Furthermore, detailed experiments of this kind using the International Standards for Diphtheria and Tetanus Antitoxins gave the results shown in Tables I and II. The values of logK were calculated as shown in Equation (3) (page 171).

**TABLE I. ACCELERATED DEGRADATION TEST OF INTERNATIONAL STANDARD FOR DIPHTHERIA ANTITOXIN**

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time</th>
<th>Remaining activity (percentage of original)</th>
<th>LogK (formula 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>56</td>
<td>22 weeks</td>
<td>80</td>
<td>−7.9</td>
</tr>
<tr>
<td>120</td>
<td>16 hours</td>
<td>13</td>
<td>−4.5</td>
</tr>
<tr>
<td>140</td>
<td>125 minutes</td>
<td>10</td>
<td>−3.5</td>
</tr>
<tr>
<td>150</td>
<td>20 minutes</td>
<td>40</td>
<td>−3.0</td>
</tr>
<tr>
<td>150</td>
<td>40 minutes</td>
<td>13</td>
<td>−3.0</td>
</tr>
<tr>
<td>150</td>
<td>80 minutes</td>
<td>1</td>
<td>−3.0</td>
</tr>
</tbody>
</table>

**TABLE II. ACCELERATED DEGRADATION TEST OF INTERNATIONAL STANDARD FOR TETANUS ANTITOXIN**

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time</th>
<th>Remaining activity (percentage of original)</th>
<th>LogK (formula 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>56</td>
<td>8 weeks</td>
<td>85</td>
<td>−7.5</td>
</tr>
<tr>
<td>120</td>
<td>4 hours</td>
<td>30</td>
<td>−4.1</td>
</tr>
<tr>
<td>120</td>
<td>8 hours</td>
<td>8</td>
<td>−4.1</td>
</tr>
<tr>
<td>150</td>
<td>20 minutes</td>
<td>5</td>
<td>−2.6</td>
</tr>
</tbody>
</table>

As may be seen from Equation (4), a plot of logK against 1/T should produce an almost straight line, the slope of which will be determined by ΔH° and the position by ΔS°.

Fig. 1 shows such a plot of the experimental values of logK collected above. It will be seen that the expected rectilinear relationship is not
FIG. 1. EXPERIMENTAL VALUES OF \( \log K \) FOR SOME INTERNATIONAL SERUM STANDARDS

contradicted by the data. The curves drawn through the points correspond to the following calculated values:

<table>
<thead>
<tr>
<th>Preparation</th>
<th>( \Delta H^\ddagger ) (cal/mole)</th>
<th>( \Delta S^\ddagger ) (cal/mole. Degree)</th>
</tr>
</thead>
<tbody>
<tr>
<td>International Standard for Diphtheria Antitoxin (dry), in stock-ampoules</td>
<td>32 500</td>
<td>4</td>
</tr>
<tr>
<td>International Standard for Tetanus Antitoxin (dry), in stock-ampoules</td>
<td>32 000</td>
<td>5</td>
</tr>
</tbody>
</table>

These figures may be compared to known values for heat inactivation of enzymes and heat denaturation of proteins and similar substances. Some of the values reported by Stearn⁴ and Pollard³ are:

<table>
<thead>
<tr>
<th>Preparation</th>
<th>( \Delta H^\ddagger )</th>
<th>( \Delta S^\ddagger )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>35 600</td>
<td>24</td>
</tr>
<tr>
<td>Trypsin</td>
<td>40 000</td>
<td>45</td>
</tr>
<tr>
<td>Tobacco mosaic virus (dry)</td>
<td>27 000</td>
<td>0</td>
</tr>
</tbody>
</table>
The ordinate in Fig. 1 covers a very large range of deterioration rates. The logK value of $-2$ means a 90% loss of activity in less than 5 minutes, whereas the logK value of $-9$ means that more than 3 years are required for a 10% loss. Experiments can, therefore, only be carried out within these two levels. The logK level of $-10.8$ represents a very slow rate of deterioration requiring 100 years for a 5% loss in potency. It will be seen from the extrapolated curves that the four preparations concerned deteriorate even more slowly when kept below freezing point.

The rate of deterioration of the International Standard for Antidysentery Serum (Shiga) in the dry state at 56°C is shown by one point in Fig. 1 (logK = $-7.3$). If we assume the slope of the curve for antidysentery serum to be similar to the slope of the curves for the two other sera, even this least stable of the dry preparations would, during storage below $-10^\circ$C, suffer less than 1% deterioration in 1000 years. Obviously, these calculations involve the assumption that the theoretical extrapolation is valid.

*Stability tests of solutions of standards*

The solutions of the international serum standards as dispensed and distributed to other laboratories are less stable than the dry material from which they are prepared.

All solutions, except two, are made up in a 66% glycerol-saline medium. The two exceptions are the International Standard for Diphtheria Antitoxin for Flocculation Test, and the International Standards of Staphylococcus Antitoxin, which are made up in phosphate buffered saline, containing 0.01% merthiolate (a number of international diagnostic serum standards, established during the past 5 years, are distributed in the freeze-dried state, whereas the International Reference Preparations of Cholera Agglutination Sera are distributed in the fluid, undiluted state).

These samples of the International Standards are transmitted to applicants by post, and are used by the receiving laboratories for comparative measurements of potency for a period of 6 months after their dispatch. During this period, therefore, the specimens are subjected to the uncontrolled temperatures of transport, and afterwards to laboratory storage. Some specimens mailed to the tropics may well be exposed to temperatures near 40°C for short periods.

The following observations contain some evidence concerning the stability of specimens of the international serum standards in the temperature range concerned; specimens of the International Standards for Tetanus Antitoxin and Antidysentery Serum (Shiga) both made up in a 66% glycerol-saline solution were mailed to Switzerland, kept for 12 years at room temperature, and returned to Copenhagen. They had lost 28% and 35% of the original potency. A parcel containing a similar specimen of the International Standard for Tetanus Antitoxin was mailed to Mexico and
returned a year later, unopened and undelivered, from a Mexican post-office. No decrease in potency could be demonstrated.

Similar specimens of the same two International Standards were subjected, in the laboratory, to temperatures of 37°C and 56°C for a period of 8 weeks. At 37°C the tetanus specimen lost 5% in potency, whereas the dysentery specimen lost about 25%. At 56°C the deterioration of both specimens exceeded 50%.

Specimens of the International Standards for Diphtheria Antitoxin, Gas-Gangrene Antitoxin (Histolyticus), and Antidyentery Serum (Shiga), in 66% glycerol-saline were kept at 25°C for 6 months. In none of these solutions could any degree of deterioration of antitoxic activity be detected.

Llewellyn Smith found a 23% loss in potency of Standard Diphtheria Antitoxin in 66% glycerol-saline after 4 years' storage at room temperature, and a 50% loss after 9 months at 35°C.

The above observations on the deterioration of serum standards in glycerol-saline show that the rate of deterioration at room temperature is very small. The samples of the international serum standards as issued are not supposed to be used for more than 6 months after issue. Even in a laboratory where the cold storage is unreliable, it would be quite safe to use these standards when kept at room temperature, not exceeding 25°C. When held in cold storage, the potency of these standards can safely be relied upon even several years after the expiry date indicated on the labels. The observations reported also indicate that a week or even two under unfavourable conditions of transport of, say, 37°C, will not lead to a noticeable loss of potency.

Dry specimens of the serum standards, prepared by reconstituting the international standard preparations in water followed by freeze-drying, were found to be just as stable, at 37°C and 56°C, as the original dry standard preparations themselves.

A few tests carried out with samples of the International Standards for Diphtheria Antitoxin, Gas-Gangrene Antitoxin (Histolyticus), and Antidyentery Serum (Shiga) dissolved in saline with merthiolate as a preservative, showed that the stability at 37°C and 56°C was less in this medium than in the glycerol-saline medium.

The traditional way of dispensing these standards in glycerol-saline solution, therefore, ensures better protection against unfavourable transport temperatures.

Though no systematic series of tests on all international serum standards have so far been completed, we may conclude from the above observations that the specimens of these standards are protected by a very large margin from significant thermal deterioration during the period of validity assigned to them, and that stability considerations do not provide a reasonable argument for changing the established way of dispensing and distributing these preparations.
The observations mentioned, and further experiments using solutions of the International Standards for Diphtheria and Tetanus Antitoxins, gave the results shown in Tables III and IV. These results were evaluated by the same methods as described above for the dried standards and Fig. 1 shows the graphical plot of the values of logK.

**TABLE III. ACCELERATED DEGRADATION TEST OF SOLUTION OF INTERNATIONAL STANDARD FOR DIPHTHERIA ANTITOXIN**

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time</th>
<th>Remaining activity (percentage of original)</th>
<th>LogK (formula 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-4</td>
<td>6 years</td>
<td>&gt;90</td>
<td>&lt;-9.2</td>
</tr>
<tr>
<td>21</td>
<td>4 years</td>
<td>77</td>
<td>-8.7</td>
</tr>
<tr>
<td>35</td>
<td>9 months</td>
<td>50</td>
<td>-7.5</td>
</tr>
</tbody>
</table>

* After Llewellyn Smith.

**TABLE IV. ACCELERATED DEGRADATION TEST OF SOLUTION OF INTERNATIONAL STANDARD FOR TETANUS ANTITOXIN**

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time</th>
<th>Remaining activity (percentage of original)</th>
<th>LogK (formula 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>23</td>
<td>12 years</td>
<td>72</td>
<td>-9.1</td>
</tr>
<tr>
<td>37</td>
<td>8 weeks</td>
<td>95</td>
<td>-8.0</td>
</tr>
<tr>
<td>56</td>
<td>8 weeks</td>
<td>25</td>
<td>-6.5</td>
</tr>
</tbody>
</table>

Both these glycerol-saline dissolved standards can stand up to 40°C for more than a week (logK = -7.2), and to 25°C for more than 6 months (logK = -8.5), without losing more than 5% in potency.

The theoretical considerations thus fully endorse the general conclusions of the practical approach. It will be clear from Fig. 1 that the points determined at the high temperature end of the experimentally accessible range of deterioration rates are very important for the correct fixation of the slopes of the curves. In further work along these lines the inclusion must therefore be recommended of observations of the rates of deterioration at temperatures of about 100°C and higher.

**Stability of other immunological standards**

Observations as to the stability of other international immunological standards are still scarce. A few experiments on the heat resistance of the International Reference Preparations of Cholera Agglutinating Sera and
the proposed International Reference Preparations of Schick Toxin and of Diphtheria Toxoid have shown satisfactory stability. The collection of further evidence of stability of the International Standards would be greatly assisted if all pertinent observations made by laboratory workers receiving these standards were communicated to the Biological Standardization Section of the World Health Organization, Geneva. Further direct experimental investigations will be carried out along the lines given in this paper.

CONCLUSIONS

A consideration of collected observations and experimental data on the stability of some of the international serum standards shows that the original dry preparations are extremely stable. If the theoretical extrapolations from the data of accelerated degradation tests are valid, the least stable of three international serum preparations tested may undergo less than 1% loss of potency during 1000 years of storage below $-10^\circ$C. This leaves a wide margin of safety. The specimens of the glycerol-saline solutions of international serum standards distributed by parcel post are not equally stable, but they will stand up to short periods at $40^\circ$C and to some weeks at $30^\circ$C, without significant loss of potency. At room temperature, these specimens will keep unchanged during the whole 6-month period of validity assigned to them. When stored in the ice-box, the potency of these samples will not decrease demonstrably during several years. Specimens shipped in the freezer-dried state are even more stable.

None of the tests of stability is unequivocal, and taken singly few would inspire confidence. Possibly by extended "planned" versions of the parallel stability test, confidence could be attained; perhaps the complete absence of deterioration after 6 months' storage at $37^\circ$C may similarly give confidence in the stability of a preparation at $-10^\circ$C for a period of years. Nevertheless, direct proof is not furnished by this or any other test. It is only by taking all tests together, and by considering them in the light of the fact that stringent precautions are taken in storage of the standards, that confidence in the stability of the international and other standards can be attained. All the tests of all the types described above have, in the past 20 years, uniformly provided evidence of extreme stability. While, therefore, we may lament the impossibility of any direct check, and while we must not relax our efforts to extend the scope of our tests of stability, we may be sure that the assumption of stability is a reasonable one and it is on this certainty that the whole structure of biological standardization is built.

RÉSUMÉ

La qualité primordiale d'un étalon est la stabilité. Malheureusement, il n'existe pas de méthode de mesure directe de la stabilité des substances utilisées elles-mêmes comme
référence. La stabilité des étalons biologiques internationaux n’a cependant guère été mise en question, car elle repose sur des principes solides et des techniques sûres.

Les auteurs exposent les divers moyens de mesurer indirectement la stabilité des étalons biologiques, celle des sérum en particulier.

Les substances étalons doivent être préservées de tout ce qui peut favoriser une réaction chimique: chaleur, lumière, humidité, oxygène, germes microbiens.

Tous les étalons sont conservés à $-10^\circ$C. Mais cette température, provoquant la congélation pourrait, dans le cas de certaines substances liquides, causer des modifications physiques indésirables; il y aurait donc lieu de conserver de tels étaisons à $+2^\circ$C. Les virus atténués, d’autre part, peuvent s’altérer à $-10^\circ$C et il serait nécessaire de les conserver à $-70^\circ$C. Ces cas particuliers ne s’appliquent pour le moment à aucun des étaisons internationaux.

Les étalons sont conservés à l’abri de la lumière. La dessication absolue — qui résoudrait la plupart des problèmes de stabilité — est pratiquement irréalisable. Elle n’est du reste pas toujours souhaitable: une substance déshydratée à 99,9%, en effet, est tellement hygroscopique qu’il est impossible d’effectuer une pesée valable sur ce matériau, au sortir de l’ampoule. Pratiquement, la déshydratation des étalons est poussée jusqu’aux limites compatibles avec la pesée. Afin de supprimer l’action de l’oxygène, on remplit les ampoules de gaz inerte, généralement d’azote. Des conditions aseptiques de travail et le scellement des ampoules empêchent la contamination, évitée déjà en grande partie par les précautions précédentes.

Ces mesures essentielles réduisent au minimum les risques de détérioration rapide.

Un certain nombre d’épreuves permettent de vérifier indirectement la stabilité des étalons: la détérioration accélérée, la comparaison parallèle de divers échantillons provenant d’une même préparation initiale, la réponse de l’animal d’épreuve. Les auteurs décrivent l’application de ces techniques à divers étalons, tels que le sérum antidiphthérique, les sérum antigangrène gazeuse et antidysentérique, les anatoxines diphtériques et tétañique.

Il ressort de ces épreuves que les préparations originales desséchées sont très stables. En extrapolant les résultats des tests de dégradation accélérée, la préparation la moins stable de celles qui ont été soumises aux essais, conservée à $-10^\circ$C, perdrait en 1000 ans moins de 1% de son activité. Les échantillons de solutions de sérum étaisons dans le glycérol ou le soluté salin, qui sont envoyés par la poste, ne sont pas aussi stables, mais ils supportent 40°C pendant de brèves périodes et 30°C pendant quelques semaines, sans perte d’activité appreciable.

Bien que la preuve directe ne puisse en être faite, l’expérience acquise et les vérifications indirectes faites au cours des 20 dernières années indiquent que les étaisons biologiques internationaux sont extrêmement stables, et c’est sur cette assurance qu’est fondée toute la standardisation biologique.

REFERENCES
2. Llewellyn Smith, M. (1939) Quart. J. Pharm. 12, 699