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THE THERMAL STABILITY OF YELLOW FEVER VACCINES

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The assessment of yellow fever vaccine thermostability both in lyophilized form and after reconstitution were analyzed. Two commercial yellow fever vaccines were assayed for their thermal stability. Vaccines were exposed to test temperatures in the range of 8 °C to 45 °C. Residual infectivity was measured by a plaque assay using Vero cells. The titre values were used in an accelerated degradation test that follows the Arrhenius equation and the minimum immunizing dose was assumed to be 10^3 particles forming unit (pfu)/dose. Some of the most relevant results include that (i) regular culture medium show the same degradation pattern of a reconstituted 17D-204 vaccine; (ii) reconstituted YF-17D-204 showed a predictable half life of more than six days if kept at 0 °C; (iii) there are differences in thermostability between different products that are probably due to both presence of stabilizers in the preparation and the modernization in the vaccine production; (iv) it is important to establish a proper correlation between the mouse infectivity test and the plaque assay since the last appears to be more simple, economical, and practical for small laboratories to assess the potency of the vaccine, and (v) the accelerated degradation test appears to be the best procedure to quantify the thermostability of biological products.

Key words: yellow fever virus – yellow fever vaccines – flaviviruses

Yellow fever 17D vaccine is one of the most successful of live attenuated vaccines. This vaccine elicits seroconversion in over 95% of vaccinees when inoculated subcutaneously in patients with or without pre-existing antibodies to other flaviviruses. Immunization is effective for 10 years, but there are studies showing the persistence of antibodies for over 35 years (Pinheiro & Gomes, 1980; Polland et al., 1981).

Despite the recommendation to the contrary, the effectiveness of the vaccine in terms of inducing a primary antibody response in vaccinees is not impaired by the simultaneous administration of immunoglobulin (Kaplan et al., 1984). In addition, yellow fever virus vaccine may also be administered together with other live virus vaccines. The minimum immunizing dose to elicit a satisfactory immune response is routinely measured by a potency test in mice (WHO, 1957). A 10^3 mouse LD_{50} is regarded as representing the minimum immunizing dose. Individual laboratories are recommended at present to determine an adequate relationship between mouse test and plaque assays using cell cultures (WHO, 1976).

Although the prevention and control of yellow fever virus relies heavily on vaccination, present stocks worldwide are extremely low to meet an immediate need should it occur now or in the near future (Bres, 1980). Furthermore, it has been recognized in Brazil that 50% of the vaccine doses are lost after leaving the manufacturer as a result of thermal degradation (Tauil et al., 1982). There is a need, therefore, to maintain an appropriate cold-chain of the product during delivery, but often this is neither possible nor feasible.

It has recently been suggested (PAHO, 1981; 1984) that there should be renewed research on methods to characterize and standardize existing yellow fever virus vaccines, the methods of vaccine production, its stabilization, and administration of the vaccine to susceptible subjects. In the present study, the thermostability of yellow fever vaccine, both

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in lyophilized form and after reconstitution was analyzed, and the most suitable method for the measurement of thermo-lability and the prediction of decay at temperatures other than those examined experimentally was determined.

MATERIALS AND METHODS

Cell culture – The experimental work was performed using Vero (African green monkey) cell line dispersed using a mixture of trypsin and versene at a final concentration of 0.05% and 0.02%, respectively. The growth medium was 199 supplemented with 4% foetal calf serum (Gibco, USA), 2.2 g/l sodium bicarbonate, penicillin (100 IU/ml), and streptomycin (100 μg/ml).

Virus vaccines – Two commercial yellow fever vaccines were assayed for thermal stability. The Vacina Anti-Amar!lica, Biomanguinhos-FIOCRUZ, Brazil, lot 182A, 50 doses per vial, substrain 17DD (kindly provided by Dr O. S. Lopes) and Arilvax, Yellow Fever Vaccine, Wellcome Laboratory, lot B YF/1/233, 1 dose per vial, substrain 17D-204 (kindly provided by Dr J. Beale), were exposed to test temperatures in their lyophilized form. Another batch of Arilvax, lot A YF/5/108, 5 doses per vial, was reconstituted with 2.5 ml distilled water prior to exposure to test temperatures.

Thermal exposure – The lyophilized products were exposed to test temperatures of 45 °C, 37 °C and 31 °C. For reconstituted products the temperatures were 37 °C, 31 °C, 21 °C and 8 °C. All vials were kept totally immersed in water baths. Lyophilized vaccines were reconstituted with 2 ml (Biomanguinhos) or 0.5 ml (Wellcome) distilled water provided by the manufacturer prior to tritation for residual virus.

Infectivity assay – All infectivity tests were performed by a plaque assay using Vero cells based on the procedures of Madrid & Porterfield (1969) and by Mann et al. (1980). Briefly, diluted or undiluted samples were dispensed in 50 μl aliquots in triplicate wells of a 24-multiwell plastic plate (2 cm^2, Nunc, Denmark). Vero cells (5 x 10^5 cells) were added and attachment and adsorption was allowed to occur over a 3 h period at 37 °C. Medium was replaced with the same formulation containing 1.5% (w/v) carboxymethylcellulose (BDH, England). After a further 4 to 6 days incubation at 37 °C, the cells were fixed with 20% formalin and stained with crystal violet at a final dilution of 1:10,000. Plaques were counted at a magnification of 12.5 times, and virus titres expressed either as $\log_{10}$ plaque forming units (pfu)/ml or corrected to $\log_{10}$ pfu/vial.

Accelerated degradation test – After the assay for infectivity of residual virus, $\log_{10}$ titre values were subjected to linear regression analysis against the exposure time. Linear regression produced values for the degradation rate, the intercept, the slope, and the half life at the respective temperatures. The values for the degradation rate were thereafter plotted against absolute temperature according to the Arrhenius equation. Briefly, the Arrhenius equation was initially derived to describe the effects of temperature on the rates of chemical reactions, and its use is a general method of quantifying the effects of temperature on biological systems (Farrell & Rose, 1967). The equation is defined as $K = A.e^{-E/RT}$ where K, is the velocity constant for the reaction (degradation rate); T, is the temperature in degrees absolute; A, is a constant of integration that includes the number of activated molecules and their frequency of collision and a probability factor; E, is the minimum energy difference between the reactants and the products; and R, is the gas constant.

The Arrhenius law states that the rate of reaction is directly proportional to the reciprocal of the absolute temperature. Since the terms A and E in the above equation are assumed to be constants for a particular reaction, a plot of log K against 1/T gives a straight line with a slope of $-E/2.303R$. The plot produced by the Arrhenius equation generated the slope and the corrected values for the degradation rate at each test temperature. It is also possible with this method to extrapolate degradation values for predictive temperatures in an accurate manner. The corrected half life at the test or projected temperature is obtained by the ratio of $\log_{10}$ 2 (= 0.3, i.e, the time to decay half of the original value) and the respective degradation rate.

In order to calculate the time for the vaccines to reach the minimum immunizing dose, it was initially assumed that such a dose would be 10^3 pfu/dose, so that the residual value for excess of virus in $\log_{10}$, is divided by the corrected degradation rate.

TABLE I

Linear regression and data produced by the Arrhenius equation for the calculation of the thermal degradation rate of reconstituted yellow fever virus vaccine, substrain 17D-204

Linear regression				Arrhenius equation			
Test temperatures $^{o}C\text{-}1/^{o}K \times 10^{6}$	No. of values	Correlation coefficient (r)	Degradation rate (k)	Correlation coefficient (r)	Slope ($s \times 10^{6}$)	Corrected degradation rate (k)	Half life (hours)
37-3226	6	0.969	0.23171			0.18931	1.6
31-3290	5	0.999	0.08538	0.994	4559.1	0.09669	3.1
27-3333	4	0.992	0.05301			0.06157	4.9
8-3559	6	0.978	0.00617			0.00574	52.3 (2.2 days)
2-3636						0.00256	117.2 (4.9 days)
0-3663						0.00193	155.4 (6.5 days)

TABLE II

Time required at experimental and predicted temperatures for the reconstituted yellow fever vaccine substrain 17D-204, to degrade to a minimum of $\log_{10}$ 3 pfu/dose

Test temperatures $^{o}C\text{-}1/^{o}K \times 10^{6}$	Virus Content per vial	Virus Content per dose	Loss/ vial	Balance/ vial	Balance/ dose	Excess pfu/dose	Inactivation rate $\log_{10}$ pfu/day	Hours to 10^{3} pfu/dose
37-3226			0.1	5.2	4.5	1.5	0.18931	7.9
31-3290			0	5.3	4.6	1.6	0.09669	16.5
27-3333			0	5.3	4.6	1.6	0.06157	26.0
8-3559	5.3	4.6	0	5.3	4.6	1.6	0.00574	278.7 (11.6 days)
2-3636			0	5.3	4.6	1.6	0.00256	625 (26 days)
0-3663			0	5.3	4.6	1.6	0.00193	829 (34 days)

RESULTS

Thermal degradation of the reconstituted yellow fever vaccine – The results obtained with a reconstituted biological product (substrain 17D-204) after exposure to four test temperatures are shown in Tables I and II. Corrected degradation rates of 0.18931 and 0.00574 are equivalent to half lives in the range of 1.6 to 52.3 h at test temperatures of 37 ^{o}C and 8 ^{o}C, respectively. The slope value, 4559.1, was similar to that obtained with two cloned substrains (data not shown). Table II shows that the vaccine still contained the minimum immunizing dose after more than 10 days of storage at 8 ^{o}C following reconstitution. At the highest temperature used (37 ^{o}C), the vaccine was viable for almost 8 h.

Thermal degradation of lyophilized vaccines – The infectivity of two lyophilized yellow fever vaccines produced by Biomanguinhos (substrain 17DD) and Wellcome (substrain 17D-204) were examined over the same range of temperatures. Tables III to VI contain the data relevant for the Arrhenius equation and the time degradation of these vaccines to reach an assumed minimum immunizing dose of $\log_{10}$ 3 pfu/dose.

Both lyophilized vaccines had an initial pattern of degradation characterized by a rapid decline in infectivity, differing from the slower first order kinetic pattern of degradation which followed. The first component of degradation (Tables IV and VI; under "loss/vial") was calculated as the difference between the titre of unexposed virus and the value found for the intercept (zero time) in the calculation of the linear regression of the second component of degradation. The correlation coefficient values for the regression lines were statistically adequate ($p < 0.05$).

TABLE III

Linear regression and data produced by the Arrhenius equation for the calculation of the thermal degradation rate of lyophilized yellow fever virus vaccine, substrain 17DD

Test temperatures $^{o}C\text{-}1/^{o}K \times 10^{6}$	No. of values	Correlation coeficient (r)	Degradation rate (k)	"0" time intercept	Correlation coeficient (r)	Slope ($s \times 10^{6}$)	Corrected degradation rate (k)	Half life (days)
45-3145	4	0.988	0.10786	5.34			0.10569	2.8
37-3226	4	0.981	0.06585	5.78	0.995	2290.2	0.06895	4.4
31-3290	4	0.952	0.05048	5.98			0.04920	6.1
8-3559							0.01191	25.2
2-3636							0.00793	37.8
0-3663							0.00688	43.6

TABLE IV

Time required at experimental and predicted temperatures for the lyophilized yellow fever vaccine subtrain 17DD, to degrade to a minimum of $\log_{10}$ 3 pfu/dose

Test temperatures $^{o}C\text{-}1/^{o}K \times 10^{6}$	Virus content		Degradation component 1			Degradation component 2		
	per vial	per dose	Loss/ vial	Balance/ vial	Balance/ dose	Excess pfu/dose	Inactivation rate $\log_{10}$ pfu/day	Days to 10^{3} pfu/dose
45-3145			0.69	5.34	3.64	0.64	0.10569	6.1
37-3226			0.25	5.78	4.08	1.08	0.06895	15.7
31-3290			0.05	5.98	4.28	1.28	0.04920	26.0
8-3559	6.03	4.33	0	6.03	4.33	1.33	0.01191	117.7 (3.7 months)
2-3636			0	6.03	4.33	1.33	0.00793	167.7 (5.6 months)
0-3663			0	6.03	4.33	1.33	0.00688	193.3 (6.4 months)

The 17DD substrain vaccine showed a lower value for the slope compared to the 17D-204 substrain (2290.2 versus 4019). Corrected degradation values ranged from 0.10569 to 0.00688 (17DD vaccine) and from 0.23698 to 0.00196 (17D-204 vaccine), at test and predictive temperatures of 45 ^{o}C and at 0 ^{o}C, respectively. The initial loss as represented by the first component of degradation was higher in the Biomanguinhos product but less at the lower temperature of 31 ^{o}C (11% versus 7% and 0.8% versus 4%, respectively). The vaccine produced by Biomanguinhos (substrain 17DD), presented as a 50 dose/vial, retained a minimum immunizing dose of $\log_{10}$ 3 pfu/dose for 15 days at a test temperature of 37 ^{o}C (Table IV). The Wellcome lyophilized product (substrain 17D-204), in the presentation of one dose/vial, was still viable for use up to at least 6 days at 37 ^{o}C (Table VI).

DISCUSSION

The degradation of viruses and their biological products can be assayed by several different methods. However, in order to quantify the effect at low temperatures the process is unsatisfactory as a long time is frequently needed to produce a suitable result. The accelerated degradation test, initially described by Jerne & Perry (1956), circumvents the need for lengthy experiments by assessing the degradation of biological products after storage at relatively high temperatures. The degradation rates are then extrapolated to give an estimate of degradation at lower temperatures. This negates the need to expose virus for prolonged periods of time at very low temperatures.

Thermal degradation follows the Arrhenius relationship with respect to absolute tempera-

TABLE V

Linear regression and data produced by the Arrhenius equation for the calculation of thermal degradation rate of lyophilized yellow fever virus vaccine, substrain 17D-204

Test temperatures $^{o}C\text{-}1/^{o}K \times 10^{6}$	No. of values	Correlation coefficient (r)	Degradation rate (k)	"0" time intercept	Correlation coefficient (r)	Slope ($s \times 10^{6}$)	Corrected degradation (k)	Half life (days)
45-3145	4	0.985	0.22524	3.92			0.23698	1.3
37-3226	4	0.996	0.12565	3.94	0.989	4019	0.11199	2.7
31-3290	5	0.995	0.05808	4.07			0.06194	4.8
8-3559				0.00514				58.4
2-3636				0.00252				119.0
0-3663				0.00196				

TABLE VI

Time required at experimental and predicted temperatures for the lyophilized yellow fever vaccine substrain 17D-204, to degraded to a minimum of $\log_{10}$ 3 pfu/dose

Test temperatures $^{o}C\text{-}1/^{o}K \times 10^{6}$	Virus content		Degradation component 1			Degradation component 2		
	per vial	per dose	Loss/ vial	Balance/ vial	Balance/ dose	Excess pfu/dose	Inactivation rate $\log_{10}$ pfu/day	Days to 10^{3} pfu/dose
45-3145			0.31	3.92	3.62	0.62	0.23698	2.6
37-3226			0.29	3.94	3.64	0.64	0.11199	5.7
31-3290			0.16	4.07	3.77	0.77	0.06194	12.4
8-3559	4.23	3.93	0	4.23	3.93	0.93	0.00514	180.9 (6.0 months)
2-3636			0	4.23	3.93	0.93	0.00252	369.0 (12.3 months)
0-3663			0	4.23	3.93	0.93	0.00196	474.5 (15.8 months)

ture. Vaccine potency decreases linearly with time at any one temperature, and the rates of decline obtained at different temperatures may be predicted by substituting the appropriate values into the Arrhenius equation. The use of degradation tests thus provide an accurate and rapid test for the prognosis of longevity of viruses and other biological products. This method has been successfully used for several viral and nonviral products (Greiff & Rightsel, 1965; Peetermans et al., 1973; Burfoot et al., 1977; Kurokawa et al., 1979; Allison et al., 1981).

A comparison of recent findings on yellow fever vaccine stability with early reports does not provide a useful retrospective analysis, mainly as a result of the changes the vaccine has undergone over many years. These changes have also involved the establishment of a virus seed, methodology of preparation, the generation of an avian leukosis free seed virus, and improvements in the formulation of the dilution medium. In addition, critical differences have evolved in the methodology of the assay used for measuring residual infectivity (Burruss & Hargett, 1947; Hahn & Bugher, 1953; Cannon & Dewhurst, 1955; Robin et al., 1971; Burfoot et al., 1977; Lucasse & Visser, 1978; Tannock et al., 1980; Barme & Bronnert, 1984; Lopes et al., 1987, 1988a, b).

The stability of a reconstituted yellow fever virus vaccine substrain 17D-204 is shown in Tables I and II. This product has a half life of 1.6 h at 37 ^{o}C and a predictable half life of more than six days if kept in an ice bath. The recommendation from vaccine manufacturers is that following reconstitution, vaccines should be discarded within 1 h. However, there is no published data to support this procedure. The World Health Organization (WHO, 1971) initially suggested that reconstituted vaccine should be discarded after 1 h in the interest of sterility. If conditions permit sterility of the product to be maintained with be use of

multiple syringe and needles, however, a considerable portion of vaccine material may be wasted, and the data shown here indicate that reconstituted vaccine can be kept for a whole working day provided suitable refrigeration is available. This should also considerably alleviate the problem that the stock of vaccine in the world is not at an optimal level should an extensive outbreak of yellow fever occur (PAHO, 1984). Assuming a hypothetical level of $\log_{10}$ 3 pfu/dose as the minimum immunizing dose, a batch of 5 doses/vial of the reconstituted vaccine would remain viable for almost 8 h, even if maintained at 37 °C.

The degradation rate of the reconstituted product was not significantly different from that of the cloned substrains of yellow fever virus stocks (data nor shown), maintained in 199 medium plus 4% foetal calf serum. The slight difference shown for the vaccine may be attributed to the presence of thermostabilizers (Burfoot et al., 1977).

The information obtained with the lyophilized vaccines showed that the 17D-204 vaccine has a superior formulation, probably due to both the presence of stabilizers in the preparation and the modernization in the vaccine production (Tables III and V). The Arilvax vaccine (substrain 17D-204) is the result of the first attempt to produce a stabilized yellow fever virus vaccine (Burfoot et al., 1977; Freestone et al., 1977), whereas the Biomanguinhos vaccine (substrain 17DD) is still prepared with few modifications from an original protocol used in the 1950's (Penna, 1956). In spite of the improvements shown by the 17D-204 vaccine, it is still a product that cannot be successfully compared to other attenuated virus vaccines, for example measles vaccines (Allison et al., 1981).

The use of an accelerated stability test on lyophilized measles vaccines revealed that lyophilized products showed two mechanisms of virus degradation (Allison et al., 1981; Mann et al., 1983). The presence of the two components of degradation was also detected in these studies with both yellow fever virus vaccines. Nevertheless, the stability test clearly demonstrated that differences existed between a non-stabilized and a stabilized product. If one assumes a hypothetical minimum immunizing dose of $\log_{10}3$ plaque forming units, the predictive values of degradation for the particular batches of vaccines are clearly different; at 0 °C, the lyophilized 17DD product could be safely kept for more than 6 months whereas the 17D-204 vaccine may be stored for a period almost three times longer whilst still retaining an immunizing capacity (Tables IV and VI). However, the accelerated degradation test should be analyzed with caution when considering predictive values below freezing point, although the thermostability of vaccine is not substantially affected at low temperatures (Burruss & Hargett, 1947; Peetermans et al., 1973; Burfoot et al., 1977; Lucasse & Visser, 1978).

There is currently a demand to improve the formulations of vaccine diluents but refrigeration and the availability of a cold chain are still important factors in the distribution of vaccine in the field. In the absence of proper refrigeration facilities, vaccine preparations should contain either larger amounts of virus per dose or more efficient cryoprotectors, or both. This would maintain vaccine viability until delivery. Such effort has been clearly attempted by one of the manufacturers (Lopes et al., 1988a). It is also important that a proper correlation be established between the mouse infectivity and the plaque assay for yellow fever virus (Seagroatt & Magrath, 1983). It would be more simple, economical, and practical for small virus laboratories to assess the potency of the vaccine using a plaque assay rather than maintaining an animal colony. Furthermore, this would obviously require a change in the concept of the minimum immunizing dose from the classical 50% mortality end point (LD_{50}) to a more specific unit measured in pfu/dose.

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