CHAPTER II

MATERIALS AND METHODS

1. Materials

1.1 Rabies Viruses Two freeze dried master seed rabies viruses were obtained from World Health Organisation, Geneva, Switzerland. Viruses were stored and distributed in ampoules.

1.1.1 PV-11, strain of Pasteur Rabbit Fixed Rabies Virus (also designated as the Pitman-Moore strain). used for vaccine production

1.1.2 CVS-11, mouse brain strain of fixed rabies virus, for challenge in potency test and for vaccine production (39,42).

1.2 Third international reference standard Vaccine, established in 1978, was stored and distributed in ampoules containing freeze-dried rabies vaccine, prepared in human diploid cells and inactivated with beta-propiolactone. There is an activity of 10 IU/ampoule, obtained from International Laboratory for Biological Standards, Statens Seruminstitut, Copenhagen.

1.3 Experimental animals : Mice about 10-12 gm at the same bleed and the same age

1.4 Chemicals

Beta-propiolactone	(Sigma Chemical , USA)
Dextran	(BDH, England)
Disodium hydrogen phosphate	(May and Baker, England)
Lactose	(BDH, England)
Phenol	(GPO, Thailand)

Potassium dihydrogen phosphate	(May and Baker, England)
Sodium chloride	(Merck, Denmark)
Sodium dihydrogen phosphate	(Merck, Denmark)
Sodium hydroxide	(Merck, Denmark)
Sucrose	(Difco laboratories, USA)
Thiomerosal	(Merck, Denmark)

1.5 Main apparatus for vaccine production

Bone cutting forceps	(VWR scientific, USA)
Brain homogenizer	(Silverson, England)
Brain suction with negative pressure pump	(General electric, USA)
Lyophilizer	(Leybold, GT-II, Germany.)
Refrigerate centrifuge	(ICE model B-20, USA.)

1.6 Reagents

1.6.1 Normal saline for injection

1.6.2 Sterile phosphate buffer saline solution pH 7.2 for vaccine production

NaCl	5.85	gm.
$NaH_2PO_4 . 2H_2O$	6.24	gm.
NaOH	1.62	gm.
Water qs.	1,000	ml.

1.6.3 Sterile phosphate buffer saline solution pH 7.4, diluent in vaccine potency test.

$Na_2HPO_4 . 12H_2O$	$\frac{1}{15}$ M.	85	ml.
KH_2PO_4	$\frac{1}{15}$ M.	15	ml.
Normal saline	qs	1000	ml.

1.6.4 4 % Dextran in normal saline

1.6.5 7.5 % Lactose in normal saline.

1.6.6 7.5 % Sucrose in normal saline.

1.6.7 4 % Thiomerosal in water.

2. Methods

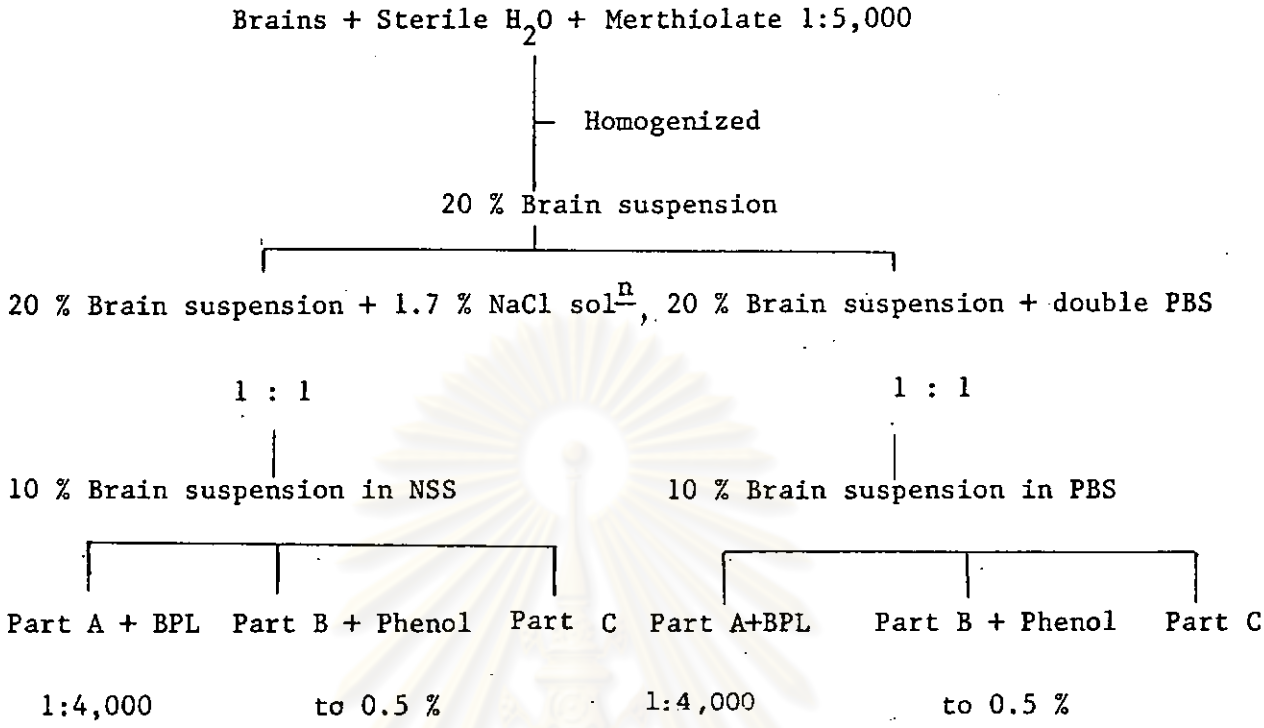
2.1 Preparation of stock working virus

From master seed virus (PV-11 or CVS-11), reconstituted and inoculated intracerebrally 0.03 ml. to mouse brain. The brains were taken when mice showed complete paralysis in the day 6-14, then homogenized them to obtain 20 % suspension with sterile water, and kept it for stock working virus. PV-11 was other step intracerebrally inoculated to rabbit with 0.25 ml. of 10 % suspension. The rabbit brain was harvested, when rabbit showed conplete paralysis in the day of 6^{th}, then homogenized to obtain 20 % suspension, this stock virus was kept for vaccine production at $-70^{o}C$ or below.

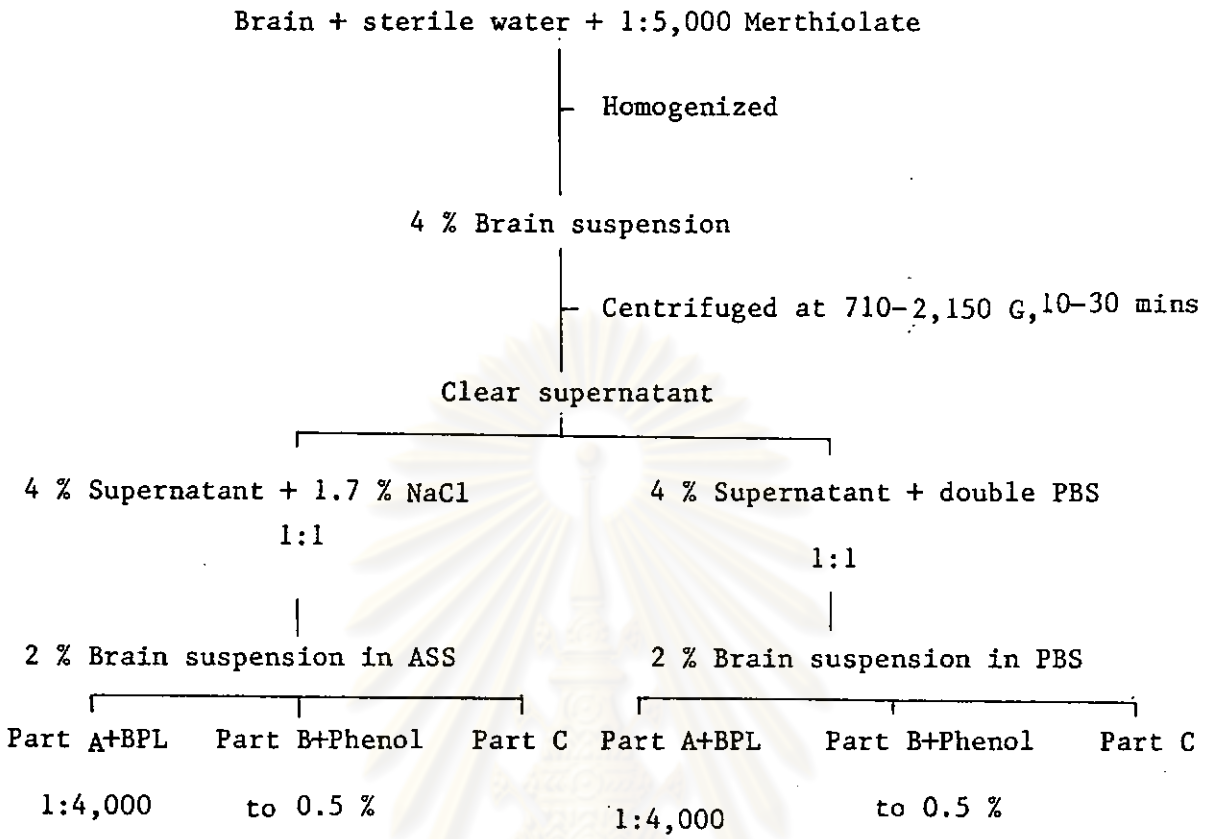
2.2 Preparation of laboratory reference standard rabies vaccine, from PV-11 that had already developed in rabbit brain, homogenized in sterile sucrose phosphate buffer saline pH 7.4 to obtain 5 % suspension, then inactivated with BPL, 1.4,000, at $37^{o}C$ 1 hr. then the inactivated suspension was kept at $4^{o}C$ 48 hr. to complete BPL hydrolysis, subdivided the suspension 10.0 ml. in 20 ml. vials, lyophilized product in lyophilizer, the product was calibrated potency with international reference standard, by NIH method.

3. Vaccine preparation

3.1 Semple Vaccine from 20 % stock rabbit brain was thawed and diluted to 10 % v/v with sterile water for injection then was inoculated intracerebrally 0.5 ml. to sheep brain, when sheeps showed marked sigh of paralysis, harvested the brains, and then prepared vaccine as diagram below.



3.2 Suckling mouse brain vaccine, from mouse working stock virus (20 % PV-11 CVS-11) diluted to 10^{-3} with 2 % normal horse serum, then inoculated intracerebrally 0.02 ml. to suckling mice not older than 4 days. Approximately 96 hours, harvested the brain with sucting apparatus after all mice showed complete paralysis, all mice were disinfected with 10 % v/v chlorhexidine and 5 % v/v phenol for 5 and 1 minutes respectively befor suction.



Note ; The Inactivation methods were done as follows;

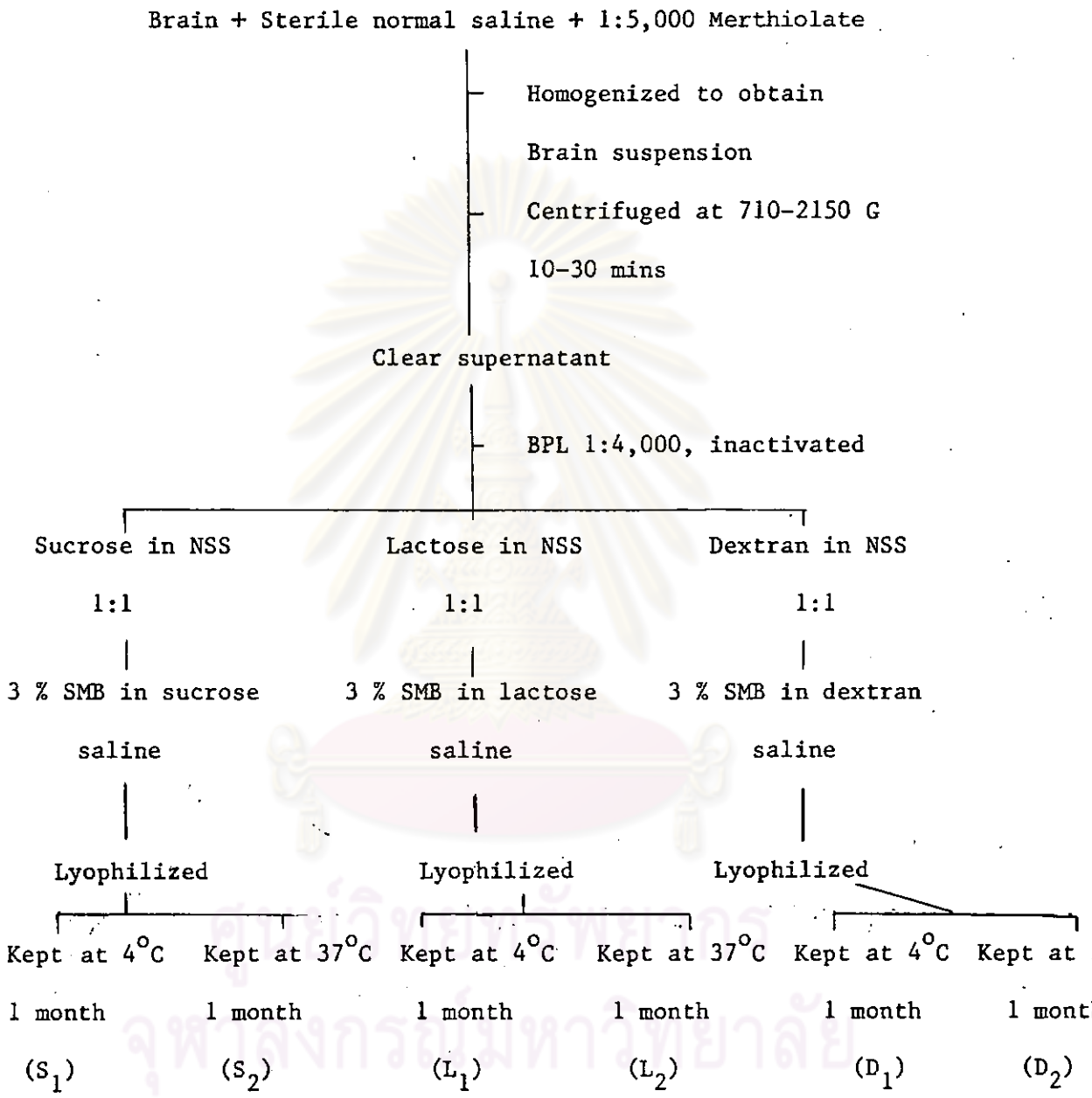
A. Beta-propiolactone (BPL) was added to brain suspension 1:4,000, incubated at 37°C for an hour, then kept at 4°C 48 hrs for complete BPL hydrolysis.

B. Phenolized Inactivation ; phenol was added to brain suspension to obtain phenol concentration of 0.25 % incubated at 37°C 48 hours with continuous stirring.

C. Heat Inactivation, the brain suspension was incubated at 37°C for 7 days

All vaccines were kept at 4-8°C, 11 days before testing.

3.3 Lyophilized suckling mouse brain vaccine ; vaccines were prepared as the diagram below



4. Vaccine Testing

4.1 Testing of virus infectivity of the pooled brain suspension was determined by intracerebrally inoculation of serial tenfold dilution into mice weighed 12-14 gm. 0.03 ml. titres ranging from 10^{-5} to 10^{-7} were considered acceptable.

4.2 Testing of virus residue, the vaccines that kept at 4-8^{o}C for 11 days after inactivating, it must be tested for virus residue, by diluting the vaccine to be 10^{-2} and 10^{-3}. Each dilution was administered intracerebrally (0.03 ml) to 3 mice weighting 10-12 gm. Observation was continued for 14 days, the vaccine should be free of residual virulence, before vaccine potency test.

4.3 Potency test. Al vaccines were tested potency by NIH method, running pararell with laboratory reference standard.

4.3.1 Diluted vaccines to dilution range of 1:5, 1:25, 1:125 and 1:625.

4.3.2 Injected each dilution intraperitoneally 0.5 ml. to 16 mice, weighing 10-12 gm. Two doses of vaccine were given to each mouse one week apart.

4.3.3 All immunized mice were challenged on the day of 14th after the first dose immunization, with CVS which was diluted in the range of 5-50 LD_{50} with 2 % normal horse serum,

Observed test, 2 % SMB vaccines from the first potency test were tested for stability by accelerate degradation method (43), by incubating at 37^{o}C for 12 days.

5. Calculation

The method of Reed and Muench were used for calculation of 50 % end point (32) and express the potency in International Unit by calculation using method from leaflet of WHO (30)

Example :

Dilution of vaccine	Number of mice	Survived	Died	Camulative total		Percentage mortality
				Survived	Died	
1:5 (10^{-7})	16	10	6	19	6	6/25 =24
1:25 ($10^{-1.4}$)	16	8	8	9	14	14/23=61
1:125 ($10^{-2.1}$)	16	1	15	1	29	29/30 =97

In this example the dilution factor is 5 and the starting point dilution (showing a mortality next below 50 % is 1:5 calculation the "Difference of Logarithms" from the formula).

$$\frac{\text{50 \% -(Mortality next below 50 \%)}}{\text{(Mortality next above 50 \%)-(Mortality next below 50 \%)}} \times \text{Logarithm of dilution factor}$$

$$\frac{50 - 24}{61 - 24} \times 0.699 = 0.491$$

Log (Reciprocal of 50 % endpoint) = Log (Reciprocal of starting point dilution)+ "Difference of logarithms"

$= 0.699 + 0.491$

$= 1.2$

50 % End-point dilution $= 10^{-1.2}$ (1:16)

And potency of vaccine = IU/ml. of reference standard vaccine x

$$\frac{\text{Reciprocal dilution of unknown vaccine}}{\text{Reciprocal dilution of standard vaccine}}$$

Suppose that 50 % and point dilution of standard vaccine = $10^{-1.4}$

And 1 ml. of standard vaccine = 1 IU.

$\therefore$ Potency of vaccine $= 1 \times \frac{10^{1.2}}{10^{1.4}}$ IU/ml.

$= 1 \times 10^{-0.2}$ IU/ml. $= 0.63$ IU/ml.