den erhitzt. Nach Erkalten wurde der Niederschlag abgesaugt und nach Waschen mit Wasser papierchromatographisch auf Whatmann 3 MM-Bogen gereinigt (2 mg II pro Bogen). Elution der Zonen mit Methanol bei 20° unter Durchlaufen. Ausbeute 4,05 mg (II). Meßergebnisse Tab. 2.

Gabe von I an Kichererbsenpflanzen

3,87 mg I wurden in 30 cm³ Wasser gelöst und die Lösung 30 10 Tage alten Pflanzen ohne Wurzeln unter Belichtung während 60 Stdn. zugeführt¹⁶. Die Aufarbeitung der Pflanzen und die Isolierung der Isoflavone erfolgte wie beschrieben¹⁶. Ausbeute 8,10 mg Biochanin-A und 2,95 mg Formononetin. Die von der Pflanze nicht aufgenommene Aktivität wurde im Rückstand bestimmt.

Gabe von I und II an Kichererbsenpflanzen

1,1 mg I plus 0,42 mg DL-Phenylalanin-(1-¹⁴C) wurden in 15 cm³ Wasser gelöst. 1,22 mg II wurden in 15 cm³ Wasser mit 0,5 cm³ n/100-NaOH in das wasserlösliche Mono-Natriumsalz überführt und 0,42 mg DL-Phenylalanin-(1-¹⁴C) zugefügt. Versuchsdauer 48 Stun-

den. Die Isolierung und Reinigung der Isoflavone erfolgte wie im vorigen Versuch. Ausbeuten: Versuch 1. 4,8 mg Biochanin-A und 1,88 mg Formononetin. Versuch 2. 4,4 mg Biochanin-A und 1,55 mg Formononetin.

Radioaktivitäts-Messungen

Die Messung der Radioaktivität erfolgte in der Gasphase nach SIMON und Mitarbb.²⁶. Die Zählausbeute betrug 70% für ¹⁴C und 50% für T. Alle angegebenen Meßwerte sind auf absolute Werte umgerechnet.

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²⁶ H. SIMON, H. DANIEL u. J. F. KLEBE, *Angew. Chem.* **71**, 303 [1959].

Inactivation of Vaccinia Virus by gamma radiation

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The influence of the purification on the inactivation of Vaccinia Virus (VV) by gamma radiation has been studied. Differential centrifugation and sucrose density gradient centrifugation have been used. Purification modifies the VV compound survival curve previously published^{1, 2}.

The possibility that multiple reactivation might be the reason of compound survival curve when crude virus preparations were irradiated has also been studied, but the experiments made give negative results.

Even with the best method of purification applied, the target volume of about 10⁻¹⁷ cm³ for VV calculated from the irradiation experiments is very small compared to the volume of the virus obtained with the sedimentation methods.

A two component exponential curve has been described¹⁻² relating Vaccinia Virus (VV) survivors to gamma radiation dose. If we assume that "target theory" works, it seems possible to expect that the virus population has heterogenous targets, or the suspension medium acts as a protector or there is in this case multiple reactivation as described for VV irradiated with ultraviolet light³. In this communication we present the results of experiments related

with the last two hypotheses. It has been found that purification decreases the 37% dose, *D*₃₇, of the radioresistant component and that multiple reactivation does not account for the deviation observed from the simple exponential law. Even in the case of the best purification obtained in this work, the sensitive volume of VV calculated from radiation curves differs from the size of VV DNA calculated by sedimentation methods⁴.

¹ R. PALACIOS, G. CONTRERAS, R. ESPEJO, R. JIMÉNEZ, A. OHLBAUM, and J. TOHÁ, *Biochim. biophysica Acta* [Amsterdam] **68**, 149 [1963].

² J. D. FRIESEN, D. SANKOFF, and L. SIMINOVITCH, *Virology* **21**, 411 [1963].

³ P. ABEL, *Virology* **17**, 511 [1962].

⁴ W. K. JOKLIK, *Virology* **18**, 9 [1962].

Material and Methods

Virus strains. The following pox virus strains were used: vaccinia Lancy strain, rabbit pox-Utrecht (RP) and Lederle 7 N. The last two strains were a gift from Dr. F. FENNER.

Growing of viruses. Chorioallantoic membranes of 11 days old chick embryos (CAM) were inoculated with 10^4 to 10^5 pock-forming units (PFU) of virus by the method of WESTWOOD et al.⁵. The eggs were incubated for 48 hours at 37 °C. The membranes were then cut out, rinsed and stored at -20 °C.

Titration of infectivity. Irradiated virus and control virus were titrated on CAM using the method of WESTWOOD et al.⁵.

Plaque counts were made in chick embryo fibroblasts (CEF) using cultures of monolayer plates. The technique followed was that of ABEL³. The number of pocks or plaques counted were in each case chosen to give a final deviation no greater than 10%.

Irradiation characteristics. The viruses were irradiated suspended in inactivated 10% horse serum (60 °C for 30 min) or in buffer tris 0.001 M p_H 7.2. The γ radiation of a Cs^{137} source was used. The total doses applied started from 1×10^4 R to 9×10^5 R. The dose rate was of the order of (200 ± 10) R/minute. This was measured using Landsverk dosimeters and by chemical dosimetry using the method given in HUTCHISON and POLLARD⁶.

Purification methods. Two methods of purification were used:

a) a partial purification consisting in a differential centrifugation: after homogenization, the virus suspended in buffer tris 0.001 M was centrifuged at 750 g for 20 min and the supernatant, centrifuged at 1400 g for 30 minutes. The pellet was resuspended in horse serum or in buffer tris 0.001 M.

b) the other method was extraction of virus with genetron from confluent CAM, centrifugation through 36% sucrose and banding in sucrose gradient, following the technique of JOKLIK⁴.

Infective Center Assays. CEF were used in infective center assays using the technique of ABEL³. In this paper no absolute multiplicities of infection are given, only relative input multiplicities are estimated.

Computation methods. All the curves have been fitted by the least squares method in a digital computer of the University of Chile. The parameters which appear in the figures are the mean values of the experiments indicated in the figures. The errors quoted are the standard deviations and in the case of the D_{37} the errors were calculated by the computer by propagation of the errors in the inactivation doses and in the survival ratios.

Experimental Results

Influence of the purification and the suspension medium in the inactivation of VV by gamma radiation

When VV is partially purified by differential centrifugation (as indicated in the Methods) and after it is irradiated suspended in horse serum 10%, the irradiation inactivation curve (Fig. 1) does not

Fig. 1. Survival curve of VV irradiated in inactivated horse serum 10 per cent. The curve corresponds to one representative experiment. The parameters in the equation are the mean values of ten experiments.

present any significant difference with the inactivation curve of VV crude preparations previously published¹⁻². The equation representing the inactivation curve is

$$\frac{N}{N_0} = 0.77 \exp\left(-\frac{D}{2.4 \times 10^4}\right) + 0.23 \exp\left(-\frac{D}{2.6 \times 10^5}\right)$$

where $\frac{N}{N_0}$ is the survival ratio and D the irradiation dose. This curve was obtained for the three strains of viruses used and when the titrations were done in MCA or in CEF. The values for the D_{37} doses are mean values of ten experiments.

When VV was suspended and irradiated after partial purification in buffer tris 0.001 M, p_H 7.2 the resulting curve of inactivation (Fig. 2) shows a significant difference in the D_{37} for the radioresistant component, which is $(7.2 \pm 3.0) \times 10^4$. This D_{37} is the mean value of five experiments. When VV non irradiated and suspended in buffer tris in the same conditions as mentioned, is maintained at the irradi-

⁵ J. C. N. WESTWOOD, P. H. PHIPPS, and E. A. BOULTER, J. Hyg. 55, 123 [1957].

⁶ F. HUTCHISON and E. POLLARD, Methods in Radiobiology, vol. I, 39 [1961].

Fig. 2. Survival curve of VV irradiated in buffer tris 0.001 M. The curve corresponds to one representative experiment. The parameters in the equation are the mean values of five experiments.

ation temperature and in different virus concentration, there is not any change in the virus, showing that the modification introduced by the buffer is due to irradiation.

Fig. 3. Survival curve of VV irradiated in horse serum 10% after extraction with genetron. The parameters are the mean values of five experiments.

Fig. 3 shows the inactivation curve after extraction of VV virus from MCA with genetron⁴. It was obtained after irradiation of VV virus in horse serum 10 per cent. The equation of inactivation is:

$$\frac{N}{N_0} = 0.94 \exp\left(-\frac{D}{4.9 \times 10^4}\right) + 0.06 \exp\left(-\frac{D}{2.1 \times 10^5}\right)$$

When VV is purified following Joklik's method, and the band, which shows the coincidence between optical density at 260 mμ and PFU, is irradiated suspended in serum 10%, it gives the curve shown in Fig. 5. This curve is of the same type as when the

virus is titrated in MCA or in CEF. The homogeneity of VV virus obtained in sucrose density gradient is shown in Fig. 4.

Fig. 4. Homogeneity of VV after purification in sucrose density gradient. ○ Optical density at 260 mμ per ml. + PFU × 10⁻⁶/ml. • Concentration of the sucrose gradient in per cent.

The equation for this case is:

$$\frac{N}{N_0} = 0.89 \exp\left(-\frac{D}{2.6 \times 10^4}\right) + 0.11 \exp\left(-\frac{D}{7.7 \times 10^4}\right)$$

the D_{37} of the two components being undistinguishable within the accuracy which the method gives.

Testing of the hypothesis of multiple reactivation

It is possible to suppose that when VV is irradiated in the presence of cellular debris, Figs. 1, 2, and 3, it has a compound exponential inactivation curve because there is multiple reactivation as it has been described for VV irradiated with UV light³. We have tried to test this hypothesis using the technique of infective center assays³.

When both non irradiated and irradiated VV with a total dose of 9×10^5 R of γ rays were inoculated in CEF with input multiplicities ranging from 0.6 PFU/cell to 20 PFU/cell the proportion of cells scoring as infective centers was nearly a constant in both cases. This experiment was repeated four times giving in all cases the same results.

Fig. 5. Inactivation of VV irradiated after purification in sucrose density gradient. It was irradiated in horse serum 10 per cent. The curve is of one representative experiment. The parameters in the equation are the mean values of four experiments.

The CEF were used for the multiplicity reactivation experiment, due to the fact that CAIRNS⁷ has established good topographic conditions of this size of cells for VV. We use in each experiment ultra sonicated VV in a concentration less than 10^6 PFU/ml to avoid clumping and therefore an error in the calculation of multiplicities, following the suggestion of GALASSO and SHARP⁸.

Discussion

The data obtained in this work suggest that purification by Joklik's method modifies the inactivation curve of VV irradiated with gamma radiation when it is compared to that obtained with the crude preparations. The D_{37} for the two components of the purified preparations do not differ statistically. Therefore, if free virus particles are heterogeneous⁹, it is not possible to show this conclusively with the radiation methods used.

The irradiation experiments in the two media of suspension, horse serum and buffer tris together

with the curves obtained with purified preparations indicate that cellular debris in the presence of horse serum may be responsible for the second component of the survival curve.

The data of Table I also suggest that the compound exponential survival curve for non purified γ irradiated VV cannot be ascribed to multiple reactivation.

Exp. Nr.	Non irradiated VV			VV irradiated with 6×10^5 R		
	M_i	P_i	$\frac{M_i \times 10^2}{P_i}$	M_i	P_i	$\frac{M_i \times 10^2}{P_i}$
1	5.0	20	25 ± 5	2.5	14	18 ± 4
	10	30	33 ± 5	5.0	31	17 ± 2
	20	90	22 ± 3	10	70	14 ± 1
				20	95	21 ± 2
2	1.3	20	6.7	1.3	31	4.2
	2.5	28	8.9	2.5	47	5.3
	5.0	77	6.5	5.0	59	8.5
	10	119	8.4	20	145	1.4
3	0.6	4	15	0.6	40	15
	1.2	10	12	1.2	89	13
	2.5	12	21	2.5	178	14
	5.0	38	14	5.0	311	16
4	1.2	38	3.2 ± 0.5	1.2	24	5 ± 1
	2.5	75	3.3 ± 0.5	2.5	43	6 ± 1
	5.0	160	3.1 ± 0.3	5.0	85	6 ± 1
	10	335	3.0 ± 0.2	10	166	6 ± 1

Table I. Multiple reactivation of VV assayed by the infective center technique in chick embryo fibroblasts. M_i means input multiplicity of pock-forming units per cell; P_i is total output of pock-forming units; the ratio $\frac{M_i}{P_i}$ is the proportion of cells scoring as infective centers.

If multiple reactivation were the reason for the compound exponential curve, we should expect that the ratio $\frac{M_i}{P_i}$ varies at least in factor of ten instead of being constant when M_i is varied.

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⁷ J. CAIRNS, Virology **11**, 603 [1960].

⁸ C. J. GALASSO and D. G. SHARP, Proc. Soc. exp. Biol. Med. **117**, 101 [1964].

⁹ C. J. PFAU and J. F. MCCREA, Biochim. biophysica Acta [Amsterdam] **55**, 271 [1962].