# ACTION OF 1:4-NAPHTHOHYDROQUINONE-DIPHOSPHATE AND ITS 2-METHYL DERIVATIVE ON IRRADIATED AND NON-IRRADIATED RATS WITH JENSEN SARCOMA

by

E. FRIEDMANN AND NORMAN T. J. BAILEY

*Department of Radiotherapeutics*

and

*Department of Medicine, University of Cambridge (England)*

## I. INTRODUCTION

J. S. MITCHELL[1, 2, 3] has shown in several papers that 2-methyl-1:4-naphthohydroquinone-diphosphate has, in a number of cases, significant palliative effects on human cancer.

In collaboration with (Mrs.) I. SIMON-REUSS[4] he has further demonstrated that this substance is a mitotic inhibitor in tissue cultures of chick fibroblasts, producing 50% inhibition at a molar concentration of $5 \cdot 10^{-6}$.

In the same paper evidence has been presented that the combined action of 2-methyl-1:4-naphthohydroquinone-diphosphate and ionising radiation gives apparently a mitotic inhibition greater than would correspond to the sum of the mitotic inhibitions due to the two inhibitors.

In the subsequent analysis of the mitotic action of some quinones on the growth of normal cells it has been found that 1:4-naphthohydroquinone-diphosphate, the parent substance of the hydroquinone investigated by MITCHELL, has, in tissue cultures of chick fibroblasts, an antimitotic activity a thousand times greater than its 2-methyl derivate (FRIEDMANN, MARRIAN, AND SIMON-REUSS[5]).

It seemed desirable to supplement the experiments *in vitro*, mentioned above, by experiments *in vivo*. For this purpose the action of 1:4-naphthohydroquinone-diphosphate and its 2-methyl derivative has been studied on non-irradiated rats and on irradiated rats with Jensen sarcoma.

The results were straightforward; neither 1:4-naphthohydroquinone-diphosphate nor its 2-methyl derivative has a significant action on the growth of non-irradiated Jensen sarcoma in rats, and for both substances the combined action of irradiation plus action of the substance failed to increase the effect of irradiation. But the study of this combined action presented features of interest to which we wish to draw attention.

## II. EXPERIMENTAL

The rats were bred from stock rats reared in the Biochemical Laboratory, Cambridge. They were inoculated with a Jensen sarcoma obtained from the Imperial Cancer Research Fund. 2-Methyl-1:4-naphthohydroquinone-diphosphate tetra sodium salt was the commercial preparation "Synkavit",

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supplied by Roche, Welwyn Garden City. 1:4-Naphthohydroquinone-diphosphate has been prepared and purified as described in the chemical section of this paper. The irradiation was performed on rats, anaesthetised with nembutal: A Seitz filtered, sterile solution was prepared from two capsules, each capsule being dissolved in 10 ml physiological saline solution; 0.5–0.6 ml were injected subcutaneously according to the size of the animal. The X-ray apparatus was the Maximar '220'; K.V. 220, mA 15, filter 1 mm Al.

After grafting the tumours into the rats approximately 8 days were given to the tumours to grow. When the tumours were clearly palpable the substance was applied intramuscularly in daily doses corresponding to 2 mg acid, neutralized with sodium hydroxide. 2 days later and 2 hours after the injection of the substance the tumours of the animals were irradiated with 500 r in nembutal anaesthesia, protecting the rest of the body with rubber, impregnated with lead. Only one irradiation was applied. The data giving the timing for the different experiments are collected in Time-table I and II.

At the end of the experiment the animals were killed and the weights of the tumours determined. The body weights of the animals were checked every second or third day. The weights of the animals at the time the tumours were inoculated are the "initial weights" of the tables; the "final weights" given are those when the rats were killed. The Time-tables show the variations in the different experiments. In the tables giving the results, the figures for initial weights, final weights and tumour weights are recorded in order to facilitate the control of the statistical treatment of the results obtained.

## III. RESULTS

### A. *Application of 1:4-naphthohydroquinone-diphosphate (S), 2 mg daily, to rats with Jensen sarcoma*

The experimental data fall into four main classes according to whether the substance was or was not administered, and whether the tumours were or were not irradiated. The experiments carried out to see whether 1:4-naphthohydroquinone-diphosphate (S) has an influence on the growth of Jensen sarcoma are summarised in Time-table I and Table of Results I, whilst Time-table II and Table of Results II contain the figures obtained with irradiated tumours alone and with irradiated tumours in the presence of the added substance.

TIME-TABLE I

EXPERIMENTS ON RATS WITH JENSEN SARCOMA WITHOUT AND WITH THE ADDITION OF 1:4-NAPHTHOHYDROQUINONE-DIPHOSPHATE, (S)

| No. of experiment | No. of rats | Time in days from injection of tumour | | No. of days in which 2 mg 1:4 N.H.Q.D.Ph. was injected daily intra-muscularly |
|---|---|---|---|---|
| | | to death | to application of substance | |
| I | 3 | 15 | — | — |
| | 3 | 15 | 10 | 5 |
| II | 4 | 15 | — | — |
| | 4 | 15 | 8 | 8 |
| III | 4 | 16 | — | — |
| | 4 | 16 | 8 | 8 |
| IV | 3 | 16 | — | — |
| | 4 | 16 | 9 | 7 |
| XIII | 6 | 14 | — | — |
| XIV | 6 | 18 | — | — |
| XII | 11 | 18 | 8 | 10 |

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TABLE OF RESULTS I

WEIGHT OF TUMOURS WITHOUT APPLICATION AND AFTER APPLICATION OF 1:4-NAPHTHOHYDROQUINONE-DIPHOSPHATE (S), 2 mg DAILY, TO RATS WITH JENSEN SARCOMA

| Experiment | No substance applied | | | Application of substance | | |
|---|---|---|---|---|---|---|
| | Weight of rats when injected | Weight of rats when killed | Weight of tumour g | Weight of rats when injected | Weight of rats when killed | Weight of tumour g |
| I | 217 | 250 | 26.5 | 227 | 244 | 16.4 |
| | 195 | 220 | 24.8 | 250 | 270 | 20.6 |
| | 227 | 247 | 16.5 | 221 | 246 | 18.0 |
| II | 328 | 377 | 29.9 | 265 | 284 | 16.2 |
| | 268 | 303 | 31.2 | 266 | 288 | 26.9 |
| | 251 | 279 | 20.0 | 335 | 356 | 22.5 |
| | 281 | 308 | 42.0 | 262 | 289 | 39.0 |
| III | 250 | 272 | 29.0 | 268 | 288 | 6.0 |
| | 249 | 283 | 29.0 | 249 | 284 | 22.0 |
| | 268 | 315 | 15.5 | 250 | 265 | 23.0 |
| | 281 | 294 | 16.0 | 205 | 217 | 17.0 |
| IV | 224 | 236 | 3.2 | 250 | 283 | 10.5 |
| | 241 | 283 | 29.7 | 273 | 302 | 22.7 |
| | 177 | 211 | 30.9 | 267 | 300 | 5.8 |
| | | | | 350 | 363 | 1.4 |
| XIII | 281 | 324 | 41.3 | Exp. XII | | |
| | 190 | 237 | 21.0 | | | |
| | 183 | 221 | 20.0 | 179 | 203 | 20.0 |
| | 160 | 191 | 6.6 | 195 | 212 | 22.3 |
| | 220 | 218 | 12.7 | 220 | 250 | 3.8 |
| | 148 | 180 | 3.9 | 190 | 216 | 11.1 |
| | | | | 129 | 187 | 40.5 |
| XIV | 167 | 234 | 34.7 | 179 | 177 | 0.9 |
| | 173 | 185 | 5.0 | 176 | 197 | 22.2 |
| | 145 | 184 | 23.4 | 176 | 197 | 18.7 |
| | 204 | 209 | 5.7 | 184 | 215 | 29.1 |
| | 167 | 208 | 44.1 | 194 | 241 | 40.7 |
| | 182 | 205 | 21.8 | 129 | 174 | 9.6 |

B. *Application of 2-methyl-1:4-naphthohydroquinone-diphosphate in daily doses of 2 mg to non-irradiated rats and to irradiated rats (500 r) with Jensen sarcoma*

The injection of the substance was started in both experiments (XIII and XIV) 8 days after the grafting of the tumours. The experiments lasted 10 days when the rats were killed. In experiment III the rats were irradiated once with 500 r 2 days after the beginning of the injections of the substance. The results are recorded in Table of Results III.

## IV. DISCUSSION

The experiments reported above show variations in the initial body weight, final body weight, and increase of body weight. From a graphical examination of the data it appears that the size of the tumour is not affected by these differences—at least so far as the variations occurring in the experiments are concerned.

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## TIME-TABLE II

EXPERIMENTS WITH IRRADIATED TUMOURS ALONE (500 r) AND WITH IRRADIATED TUMOURS (500 r) IN THE PRESENCE OF 1:4-NAPHTHOHYDROQUINONE-DIPHOSPHATE (S), 2 mg DAILY

| No. of experiment | No. of rats | Time in days from injection of tumours | | | Time in days from | |
|---|---|---|---|---|---|---|
| | | to death | to application of substance | to irradiation (500 r) | irradiation to death | irradiation + substance to death |
| V | 4 | 18 | — | 9 | 9 | — |
| | 3 | 18 | 7 | 9 | — | 11 |
| VII | 4 | 21 | — | 9 | 12 | — |
| | 4 | 21 | 7 | 9 | — | 14 |
| VIII | 5 | 19 | — | 9 | 10 | — |
| | 5 | 19 | 7 | 9 | — | 12 |
| XI | 5 | 16 | — | 8 | 8 | — |
| | 5 | 16 | 6 | 8 | — | 10 |

## TABLE OF RESULTS II

WEIGHT OF TUMOURS OBTAINED AFTER IRRADIATION WITH 500 r ALONE AND WITH IRRADIATION (500 r) IN THE PRESENCE OF 1:4-NAPHTHOHYDROQUINONE-DIPHOSPHATE, APPLIED IN DAILY DOSES OF 2 mg

| No. of experiment | 500 r | | | S + 500 r | | |
|---|---|---|---|---|---|---|
| | Initial weight | End weight | Weight of tumour | Initial weight | End weight | Weight of tumour |
| V | 228 | 254 | 2.4 | 240 | 265 | 17.4 |
| | 223 | 251 | 2.8 | 218 | 231 | 22.2 |
| | 220 | 237 | 4.3 | 240 | 265 | 12.3 |
| | 262 | 282 | 0.6 | | | |
| VII | 180 | 186 | 11.0 | 207 | 211 | 0.4 |
| | 200 | 227 | 0.4 | 203 | 224 | 20.0 |
| | 200 | 219 | 0.5 | 204 | 215 | 0.8 |
| | 172 | 195 | 1.2 | 207 | 231 | 3.5 |
| VIII | 260 | 272 | 0.6 | 207 | 218 | 14.8 |
| | 204 | 227 | 4.6 | 240 | 243 | 2.2 |
| | 226 | 249 | 2.1 | 266 | 277 | 1.5 |
| | 205 | 226 | 0.8 | 238 | 248 | 1.7 |
| | 223 | 241 | 0.4 | 240 | 245 | 0.7 |
| XI | 308 | 326 | 4.5 | 293 | 290 | 1.4 |
| | 275 | 283 | 5.4 | 300 | 327 | 17.3 |
| | 245 | 245 | 10.2 | 250 | 254 | 1.1 |
| | 260 | 259 | 2.1 | 240 | 225 | 0.8 |
| | 210 | 225 | 1.2 | 207 | 230 | 18.9 |

*References p. 282.*

TABLE OF RESULTS III

APPLICATION OF 2-METHYL-1:4-NAPHTHOHYDROQUINONE-DIPHOSPHATE (S'), 2 mg DAILY

| No. of experiment | No irradiation S' | | | No. of experiment | Irradiation S' + 500 r | | |
|---|---|---|---|---|---|---|---|
| | Weight of rats when tumour injected g | Weight of rats when killed g | Weight of tumour g | | Weight of rats when tumour injected g | Weight of rats when killed g | Weight of tumour g |
| XIV | 169 | 192 | 15.4 | XIII | 225 | 202 | 10.4 |
| | 165 | 242 | 40.1 | | 223 | 241 | 2.4 |
| | 190 | 218 | 14.3 | | 173 | 175 | 1.6 |
| | 193 | 228 | 20.2 | | 181 | 219 | 20.4 |
| | 138 | 152 | 15.0 | | 174 | 211 | 16.7 |
| | 191 | 212 | 9.8 | | 179 | 215 | 29.3 |
| | | | | | 145 | 156 | 11.2 |
| | | | | | 196 | 211 | 11.4 |
| | | | | | 138 | 158 | 8.7 |
| | | | | | 160 | 174 | 6.4 |

Another source of variation is the time over which the administration of the substance and irradiation has taken place. This is especially the case for the periods of administration of the substance to non-irradiated rats and to irradiated rats. With irradiated rats advantage has been taken of the well known retardation of tumour growth by irradiation. However, within each group the variation does not appear to influence the weight of the tumour.

The distribution of tumour weight (to the nearest gramme) for the four main classes of tumour growth under the influence of 1:4-naphthohydroquinone-diphosphate are shown in Fig. 1.

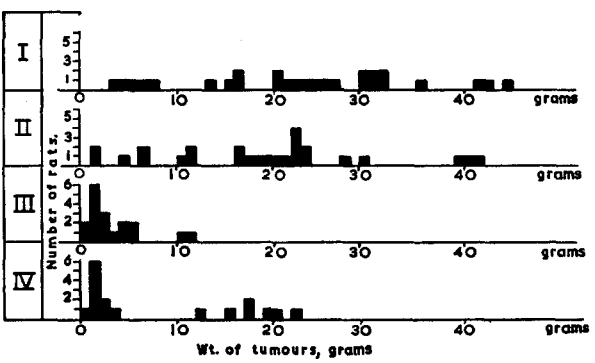


Fig. 1. Jensen sarcoma. Distribution of tumour weights (to nearest gram only)

Application of 2 mg 1:4-naphthohydroquinone-diphosphate daily to irradiated (500 r) and non-irradiated rats with Jensen sarcoma.

I. No substance applied
II. Substance applied daily (2 mg)
III. One irradiation with 500 r
IV. One irradiation, 500 r, substance applied daily (2 mg)

So far as the non-irradiated tumours go, the average weight when no substance was given was 22.48 g and 18.73 g with substance. But the difference is not significant (the difference is 3.75 ± 3.17; t = 1.18, on 50 degrees of freedom).

On the other hand, the irradiated tumours are on average markedly smaller. Here the tumours appear to fall into two distinct groups: according to whether the tumour is larger or smaller than about 9 g. When the substance is given this difference is striking, the average for the two groups being 17.6 g and 1.4 g. It is evident from the graphical data in Fig. 1 that this

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difference is significant, and this is confirmed by a t-test, performed on the square-roots of weights to make the variances in the two groups comparable; ($t = 16.4$ on 15 degrees of freedom, and $P < 0.1\%$).

The question arises whether the effect of substance on irradiated tumours is significant—although it is not in the absence of irradiation. It is doubtful whether we are entitled to regard the two tumours over 9 g in the irradiated class without substance as members of a separate "high" group or not. But even if we do this, their mean is significantly lower than the mean of the "high" group occurring when the substance is given. Another way of analysing the data is to record the numbers only of tumours above and below 9 g with and without the substance being given. This gives us a four-fold table for irradiated tumours:

| | < 9 g | > 9 g | |
|---|---|---|---|
| No substance | 16 | 2 | 18 |
| Substance | 10 | 7 | 17 |
| | 26 | 9 | 35 |

The exact treatment (R. A. FISHER[6]) of this table shows that the departure from proportionality is just significant at 5% with $P = 0.0487$. This test is rendered somewhat insensitive by its having ignored the actual magnitude of the individual tumours—and it is obvious that any attempt to do this would increase the significance of the result.

To sum up, while the effect of the substance on non-irradiated tumours has produced a small non-significant decrease in the average size of tumour, the effect on irradiated tumours is appreciable. This effect is to greatly increase the number of tumours over 9 g, and in fact seems to separate them into two distinct groups—a "low" group with an average of about 1.4 g, and a "high" group with an average of about 17.6 g. Of the 17 irradiated tumours, with substance administered, 10 fell in the former group and 7 in the latter. It would appear that the substance partially inhibits the action of the X-rays in about 40% of cases.

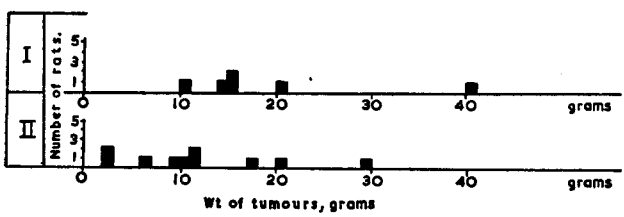


Fig. 2. Jensen sarcoma. Distribution of tumour weights (to nearest gram only)

Application of 2 mg 2-methyl-1:4-naphthohydroquinone-diphosphate daily to irradiated (500 r) and non-irradiated rats with Jensen sarcoma.

I. Substance applied daily (2 mg)
II. One irradiation of 500 r. Substance applied daily (2 mg)

The distribution of the tumour weights for 2-methyl-1:4-naphthohydroquinone-diphosphate in irradiated rats and non-irradiated rats with Jensen sarcoma is given in Fig. 2. The data are rather scanty, although what is available suggests, as before, that there is little effect on non-irradiated tumours, while the inhibition of X-ray action is even greater than before, there being 10 values, spread between 1.6 and 29.3 g.

The experiments are being continued with other tumours.

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## V. CHEMICAL SECTION

The preparation of crystalline 1:4-naphthohydroquinone-diphosphate has been improved (a) by isolating the product, obtained by the interaction of phosphorous oxychloride with 1:4-naphthohydroquinone in pyridine, as calcium salt; (b) by passing the solution of the calcium salt over a column, packed with the cation-exchanger Zeo-Karb 215, following the directions given by PARTRIDGE and his collaborators; (c) by repeating this procedure with the sodium salt, resulting from neutralising the acids liberated from the calcium salt.

The resin was prepared as described by BENDALL, PARTRIDGE AND WESTALL[7] and PARTRIDGE AND WESTALL[8]. Zeo-Karb 215 (40–60 mesh/in.) was packed in a column 70 × 1.5 cm, and the packed column (62 cm) alternately treated with 2 *N* NaOH and 2 *N* HCl before use in order to consolidate the resin (PARTRIDGE[9]). The flow was regulated to allow one drop to pass in 10".

a. *Preparation of the crude calcium salt of 1:4-naphthohydroquinone-diphosphate*

1:4-naphthohydroquinone (16 g) in ice cold pyridine (80 ml) were added under stirring to phosphorous oxychloride (30 ml) in ice cooled pyridine (300 ml) within 45 minutes. After removal from the ice the stirring was continued for half an hour at room temperature. Approximately one-third of the pyridine was then distilled off under reduced pressure. The remaining solution was cooled in ice-salt mixture to moderate the violent reaction resulting from the decomposition of the chlorides with ice water (50 ml). The so obtained fluid was concentrated *in vacuo* to a thin syrup to remove most of the added pyridine. The syrup was taken up with water (300 ml) and treated with a suspension of calcium hydroxide (100 g) in water (200 ml) until permanently alkaline. The pinkish mixture was filtered by suction, and the insoluble part well washed with water. The filtrate (1250 ml), left over night, deposited a greenish precipitate which was removed and discarded. The filtrate was brought to dryness either by distillation *in vacuo*, or, when foaming started to be troublesome, by concentration *in vacuo* over sulphuric acid. The dry residue was extracted with boiling alcohol in three portions (500, 200 and 200 ml). The two first yellow extracts were decanted, the last 200 m were sucked off. The residue, dried *in vacuo*, was buff coloured, and consisted of the crude calcium salt. The yield was 29.3 g. A corresponding yield was obtained when the quantities were changed from 1/10 to 1/100 molar proportions.

b. *Preparation of the solution of the sodium salt*

The crude calcium salt (3 g) was dissolved in water (300 ml) and after filtration passed through a column packed with Zeo-Karb 215. The filtrate was collected when acid to methyl-orange. It was clear and nearly colourless. The acid was neutralised with *N*/1 NaOH.

c. *1:4-naphthohydroquinone-diphosphate*

The sodium salt was again passed through a column packed with Zeo-Karb 215 as described. The clear, colourless filtrate was concentrated by distillation *in vacuo* to a small volume and further concentrated *in vacuo* over sulphuric acid at room temperature, preventing the substance becoming dried.

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The so obtained white product was collected, thoroughly drained by suction and pressed on porcelain; 1.2 g; m.p. 219.5°.

For analysis the substance was recrystallised from a small volume of water. (Found: C, 37.2; H, 3.1%; $C_{10}H_{10}O_8P_2$ required C, 37.5; H, 3.2%).

1:4-naphthohydroquinone-diphosphate crystallises in colourless, thin, transparent six sided plates, m.p. 220°. It exhibits blue fluorescence when irradiated with the mercury lamp. Fig. 3 gives the absorption spectrum, determined with Beckmann's spectrophotometer, and shows the shape of the crystals.

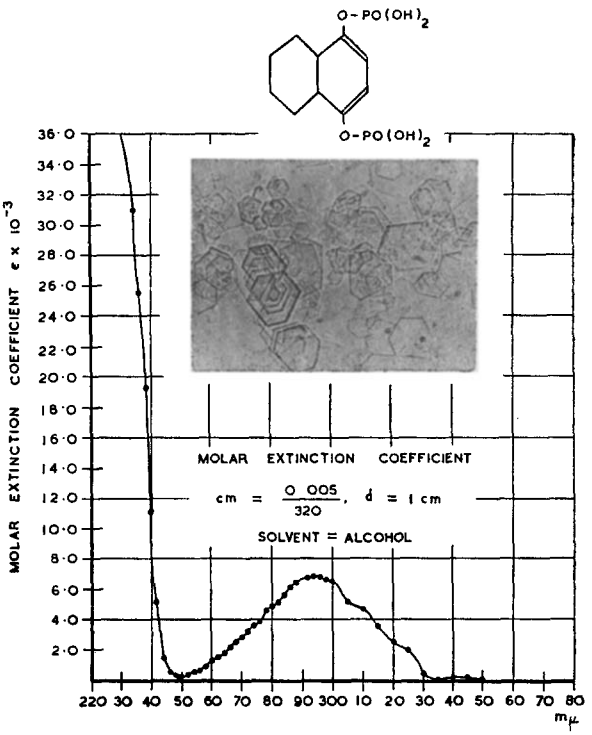


Fig. 3. 1:4-Naphthohydroquinone-diphosphate

VI. ACKNOWLEDGEMENTS

We would like to thank Mrs B. HOLMES who kindly performed the grafting of the tumours. We wish further to express our appreciation for the skilled technical assistance of Miss M. NEW, who injected the substance into the rats. One of us (E.F.) is indebted to May and Baker, Ltd., Dagenham, Essex, for financial support.

SUMMARY

1. Neither 1:4-naphthohydroquinone-diphosphate nor its 2-methyl derivative has a significant effect on the growth of a non-irradiated Jensen sarcoma in rats.

2. When 1:4-naphthohydroquinone-diphosphate is applied in combination with one irradiation of 500 r on rats with Jensen sarcoma the effect is to increase greatly the number of tumours weighing over 9 g.

3. 1:4-naphthohydroquinone-diphosphate appears to inhibit partially the action of X-rays (500 r) in 40% of the investigated cases.

4. The result in 3 is analysed statistically.

5. The preparation of pure 1:4-naphthohydroquinone-diphosphate is described.

6. The absorption spectrum of 1:4-naphthohydroquinone-diphosphate, determined with Beckmann's spectrophotometer, is recorded.

RÉSUMÉ

1. Ni la diphosphate de la 1:4-naphthohydroquinone ni celle de sa derivée 2-méthylique n'a dans les rats, une influence significative sur la croissance d'un Jensen sarcoma, non-soumis à des rayons X.

2. Si l'action de la diphosphate-1:4-naphthohydroquinone est combinée avec une irradiation de 500 r l'effet obtenu consiste dans une augmentation des tumeurs avec un poids excédant 9 g.

3. Dans 40% des cas examinés par nous, la 1:4-naphthohydroquinone-diphosphate semble empêcher partiellement l'action des rayons X (500 r).

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4. Le résultat, donné en No. 3 du résumé, a été soumis à une analyse statistique.

5. La préparation de 1:4-naphthohydroquinone-diphosphate, dans un état pur, est décrite.

6 Le spectre d'absorption de la diphosphate de la 1:4-naphthohydroquinone a été déterminé avec le spectrophotomètre de Beckmann.

## ZUSAMMENFASSUNG

1. Weder 1:4-Naphthohydrochinon-diphosphat noch 2-Methyl-1:4-naphthohydrochinon-diphosphat beeinflussen in charakteristischer Weise das Wachstum eines nichtbestrahlten Jensen-Sarkoma der Ratten.

2. Der Erfolg einer kombinierten Einwirkung von Bestrahlung mit 500 r und Applikation von 1:4-Naphthohydrochinon-diphosphat bestand in einer Vermehrung der Tumoren mit einem Gewicht von über 9 g.

3. 1:4-Naphthohydrochinon-diphosphat scheint in 40% der untersuchten Fälle die Wirkung der Röntgenstrahlen (500 r) partiell zu verhindern.

4. Das Resultat in Paragraph 3 der Zusammenfassung ist mit statistischen Methoden analysiert worden.

5. Die Darstellung von reinem 1:4-Naphthohydrochinon-diphosphat ist beschrieben.

6. Das Absorptionsspektrum von 1:4-Naphthohydrochinon-diphosphat ist mit Beckmann's Spektrophotometer ermittelt worden.

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