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# EVALUATION OF SOME UNCOUPLING AGENTS IN THE CHEMOTHERAPY OF EXPERIMENTAL CANCER: A STUDY OF THEIR DISTRIBUTION IN TUMOUR-BEARING RATS*

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**Abstract**—Four nitrophenol compounds known as uncoupling agents were administered to tumour-bearing rats to study the distribution in the body.

It was not possible to attain the concentration in the tumour, that was assumed on the basis of experiments *in vitro* to be fatal for the tumour, without endangering the life of the animal.

The half-clearance time of the compounds was in good agreement with the lipid solubility and the protein-binding capacity.

## INTRODUCTION

CLOWES and Krahl[1] showed that 2:4-dinitrophenol and related compounds interfere with cell division. Much later it was found that a fundamental action of these compounds consists of the uncoupling of oxidative phosphorylation,[2] with the result that the affected cell can no longer utilize the respiratory reaction chain for building up "energy-rich" phosphates.

The rapid proliferation of tumour tissue and the consequent high energy requirement for cell division induced us to determine whether systemic administration might inhibit the proliferation of tumour tissue more markedly than that of normal tissue.

These considerations led us first to determine for a small series of uncoupling agents the concentration required *in vitro* for uncoupling of oxidative phosphorylation in cancer cells.†

The second stage of our experiments consisted of attempts to achieve this effective concentration in experimental tumours *in vivo* by systemic administration of these uncoupling agents in doses that would be tolerated satisfactorily by the animal.

While this aim was not achieved the experiments gave some information on the distribution of these uncoupling agents in tumour-bearing rats.

Dinitrophenol, *p*-nitrophenol, dinitrocarvacrol and dinitrothymol were selected as the uncoupling agents to be used in this work. The two latter compounds were chosen

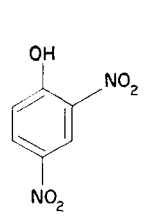
* This work was initiated by Professor Bruno Mendel, F.R.S., and carried out under his direction until his death on 23 August 1959. The paper has been prepared by his co-workers to commemorate this gifted investigator.

† Further experiments with the same uncoupling agents in these concentrations *in vitro* indicated that under certain conditions these compounds may indeed destroy the viability of tumour cells (unpublished experiments).

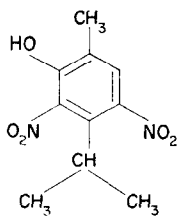
from the series of compounds studied by Clowes and Krahl[1]: dinitrocarvacrol because of its high activity, and dinitrothymol because unlike many other uncoupling agents it does not stimulate cell respiration.

## MATERIAL AND METHODS

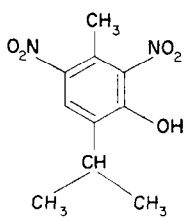
Dinitrophenol and *p*-nitrophenol were obtained commercially and purified by crystallization; the butyric ester of *p*-nitrophenol was prepared from *p*-nitrophenol and butyrylchloride in the usual way and purified by distillation. Dinitrocarvacrol and dinitrothymol were synthesized in this laboratory following a modified method of Mazzara[3]. Their formulae together with the abbreviations by which they will be referred to in this paper are shown below:



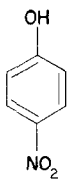

2:4-dinitrophenol (DNP)




3:5-dinitrocarvacrol (DNCa)




2:6-dinitrothymol (DNT)




p-nitrophenol (PNP)

Arachis oil or 0·21 per cent (w/v) $NaHCO_3$ was used as a vehicle for the subcutaneous administration; the latter was used when intravenous injection was required.

DNP and PNP concentrations in plasma and tissue homogenates were determined by the method of Mudge and Taggart[4]. This technique was also used for the determination of DNCa and DNT in plasma, but proved to be unsuitable for the determination of these two compounds in tumour, liver and brain homogenate. Attempts to improve the results of recovery experiments by using different organic solvents or Zander and Simmer's[5] extraction method were unsuccessful. The following method was eventually adopted. A tissue homogenate was divided into two equal portions, and 100 $\mu$g of DNCa (or DNT) was added to one. Both portions were acidified with 0·5 ml of 3 N $H_2SO_4$ and extracted with 25 ml of ether. An aliquot of 20 ml of each extract was shaken with 5 ml of 0·05 M borax–NaOH buffer solution of pH 10·6, and the absorbancy of the buffer layer measured in a spectrophotometer at a wavelength of 400 m$\mu$. The percentage recovery of the 100 $\mu$g of DNCa (or DNT) added to the homogenate was calculated from the difference between the absorbancies of the two extracts obtained, and it was assumed that the same percentage of DNCa (or DNT) had been extracted from the homogenate to which no DNCa (or DNT) was added before extraction.

Swiss mice inoculated with the Ehrlich mouse-ascites carcinoma* were used for obtaining ascites fluid and tumour cells for the experiments *in vitro*. Adult male rats from the strain bred in this institute (an inbred crossing of Piebald and Wistar rats) were used to study the absorption and elimination of the uncoupling agents. Adult

* We are indebted to Dr. G. Klein, Stockholm, for supplying us with the Ehrlich ascites carcinoma, and to Professor O. Mühlbock, Amsterdam, for supplying us with the rat mammary tumour DE 40.

male Wistar rats with the mammary tumour DE 40* were used in the experiments on the distribution of the uncoupling agents in tumour-bearing rats.

Uncoupling of oxidative phosphorylation *in vitro* was studied by measuring the effect on aerobic glycolysis. For comparison between the different uncoupling compounds, the concentrations required to bring the aerobic glycolysis up to the level of the anaerobic were compared.

The aerobic and anaerobic glycolysis of the Ehrlich mouse-ascites carcinoma were measured manometrically by Warburg's method[6] in a medium of either cell-free ascites fluid or Krebs–Ringer–bicarbonate solution,[6] to which glucose was added to a concentration of 180 mg/100 ml and the uncoupling agent in varying concentrations. The ascites fluid was used to test the extent to which binding of the uncoupling agent by protein would give protection *in vivo*. Standard volumes of 0·2 ml of cell suspension (60–80 $mm^3$ cells) and 5·0 ml of medium were employed throughout the experiments.

The affinity of the uncoupling agents for protein was evaluated by filtering a solution of each compound in ascites fluid through cellophane under a pressure of 15 cm of mercury, and measuring the concentration of uncoupling agent in the protein-free filtrate.

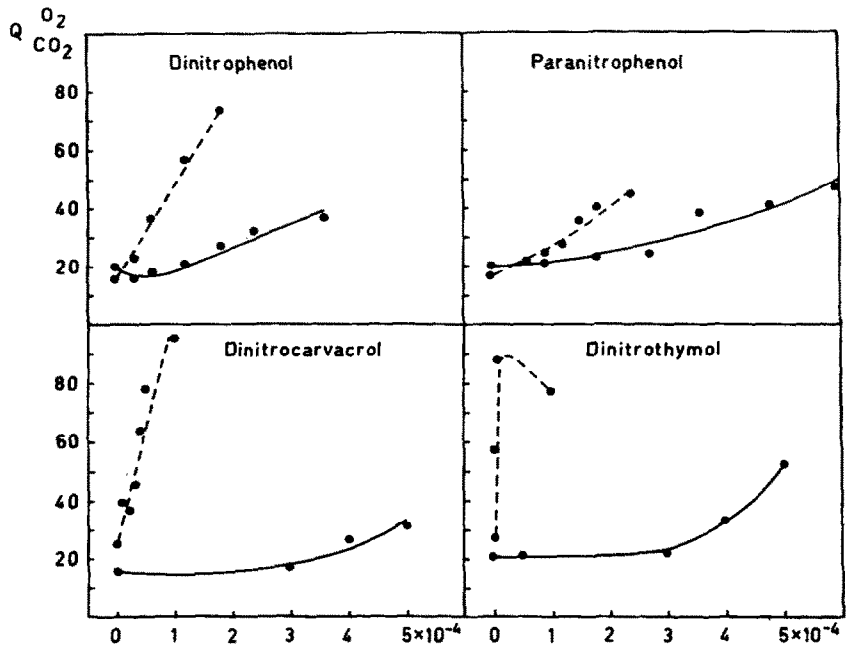



FIG. 1. The effect of four uncoupling agents on the aerobic glycolysis of Ehrlich mouse-ascites carcinoma cells *in vitro*. The molar concentration is represented along the abscissa, the rate of glycolysis along the ordinate ($Q_{CO_2}^{O_2}$;). ●– –●: aerobic glycolysis in Krebs–Ringer–bicarbonate solution. ●——●: aerobic glycolysis in cell-free ascites fluid.

## RESULTS

Fig. 1 shows the effect of the four uncoupling agents on the aerobic glycolysis of Ehrlich ascites-carcinoma cells *in vitro*, when these cells were suspended either in a Krebs–Ringer–bicarbonate solution or in cell-free ascites fluid. For the experiments *in vivo*, it was decided arbitrarily to aim at a concentration of uncoupling agent which

would bring the aerobic glycolysis up to the level of the anaerobic glycolysis in the absence of uncoupling agent, which amounted to doubling the aerobic glycolysis under our conditions. Fig. 1 shows that, in the presence of ascites fluid, this requires $3{\cdot}6 \times 10^{-4}$ M DNP, $4{\cdot}7 \times 10^{-4}$ M PNP, $4{\cdot}8 \times 10^{-4}$ M DNCa, or $4{\cdot}5 \times 10^{-4}$ M DNT.

It is clear that in the absence of ascites fluid the uncoupling agents are able to increase the aerobic glycolysis to a value considerably above that of the anaerobic glycolysis in the absence of uncoupling agent. This is in agreement with the observations of Emmelot and Bos[7] with the same tumour. These authors showed, moreover, that $10^{-4}$ M DNP more than doubled the rate of anaerobic glycolysis. The degree of stimulation of the aerobic glycolysis by $10^{-4}$ M DNP, reported by Emmelot and Bos, agrees well with the values shown in Fig. 1.

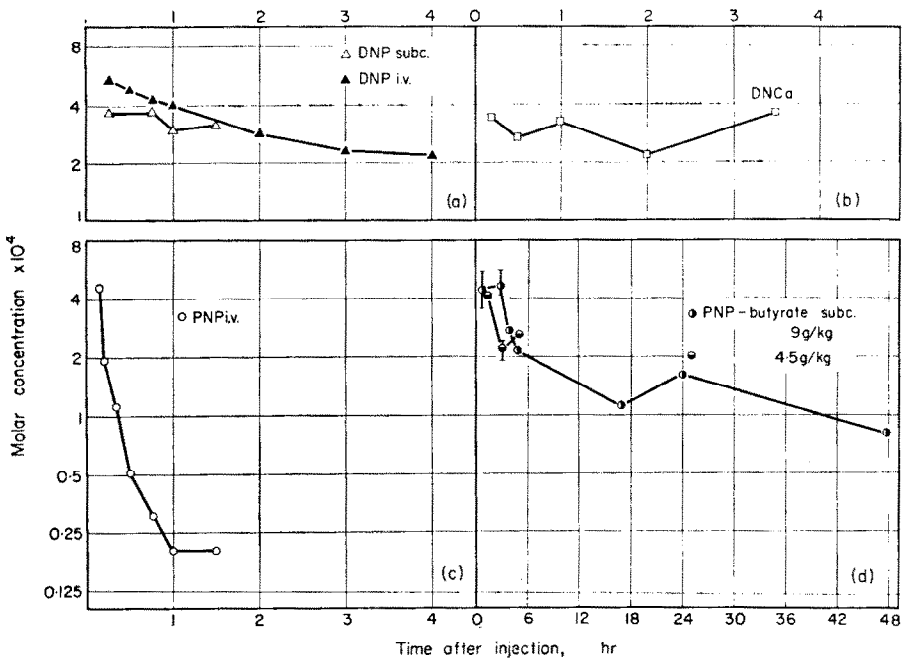



FIG. 2. The plasma level of dinitrophenol (DNP), *p*-nitrophenol (PNP) and dinitrocarvacrol (DNCa) in adult rats after parenteral administration of one dose.

(A). Individual rats were injected subcutaneously or intravenously with 20 mg DNP/kg body weight and killed at different intervals to determine the DNP concentration in the plasma. △——△ subcutaneously; ▲——▲: intravenously. (B). A similar experiment with 15 mg DNCa/kg injected intravenously. (C). The PNP concentration was studied in the same way after the intravenous administration of 75 mg PNP/kg. (D). PNP-butyrate was injected subcutaneously in a dose of 4·5(◒) or 9 g (◑)/kg, and the PNP concentration in the plasma was studied at the indicated intervals. When the concentration in the plasma was determined in two or more animals killed at the same time after injection the mean and range are indicated.

Normal rats of the same weight as those bearing tumours to be used later were injected either subcutaneously or intravenously with the uncoupling agents, and the concentration in the plasma was determined at various times after the injection, to find a dosage schedule that would yield at least the above concentrations for a reasonable time.

Tainter and Cutting[8] reported a value of 25 mg/kg for the $LD_{50}$ of DNP for the rat on subcutaneous administration, while Spencer[9] found 30 mg/kg to be the $LD_{50}$ for orally administered DNP. Therefore we chose a dose of 20 mg/kg, which was well tolerated both after subcutaneous and intravenous injection. As shown in Fig. 2 the desired minimum concentration of $3{\cdot}6 \times 10^{-4}$ M was maintained for at least 1·5 hr after a single intravenous injection of 20 mg/kg, while the half clearance is approximately 2 hr. In a subsequent experiment a male Wistar rat received an intravenous injection of 20 mg/kg, followed by four maintenance doses of 3·5 mg/kg 1, 2, 3 and 4 hr later. When the rat was sacrificed 5 hr after the initial injection a concentration of $3{\cdot}2 \times 10^{-4}$ M DNP was found in the plasma, indicating that the desired threshold concentration had been present for between 4 and 5 hr. However, when rats with the mammary tumour were treated according to this schedule the DNP concentration in the tumour itself never reached the threshold concentration, although the concentration in the plasma was in agreement with the corresponding findings in the preceding experiments.

In a pilot experiment with PNP two male Wistar rats received 100 mg/kg subcutaneously; after 25 min a PNP concentration of $3 \times 10^{-5}$ M was found in the plasma of one of the rats, while 60 min after the injection no PNP could be found in the plasma of the other animal. Fig. 2 shows the results of a second experiment, in which seven rats were given 75 mg PNP/kg body weight intravenously, and were killed at short intervals for the determination of the PNP level in the plasma. These results indicated

TABLE 1. THE *p*-NITROPHENOL (PNP) CONCENTRATION IN THE PLASMA AND IN THE TUMOUR OF RATS WITH A MAMMARY TUMOUR DURING CONTINUOUS ADMINISTRATION OF THIS COMPOUND

(Three adult male Wistar rats were anaesthetized with sodium pentobarbitone (35 mg/kg), injected subcutaneously with 100 mg PNP/kg, and immediately after connected with a motor-driven syringe delivering 180 mg PNP/kg per hr subcutaneously. The PNP was dissolved in 0·21 per cent (w/v) aq.$NaHCO_3$.)

| Rat no. | Interval between first injection and determination (min) | Concentration of PNP (M) | | Fate |
|---|---|---|---|---|
| | | In plasma | In tumour | |
| 1 | 55 | $4{\cdot}3 \times 10^{-4}$ | $3{\cdot}7 \times 10^{-4}$ | † |
| 2 | 120 | | $2{\cdot}8 \times 10^{-4}$ | |
| 3 | 300 | | $3{\cdot}7 \times 10^{-4}$ | † |

† Died.

a very rapid elimination of PNP from the plasma, the half-clearance being approximately 10 min. Attempts were then made to maintain the desired level in the plasma by injecting 100 mg PNP/kg subcutaneously, followed by a continuous subcutaneous administration of 180 mg/kg per hr with the aid of a motor-driven syringe, in rats anaesthetized with 35 mg sodium pentobarbitone per kg. The results re shown in Table 1; although an adequate concentration of PNP could be maintained in this way, the death of two out of three animals made us search for another method to reach our goal.

In a following experiment the possibility of obtaining a depot effect by using esters of PNP was investigated. PNP-acetate is very sparingly soluble, but PNP-butyrate is liquid at room temperature and may be injected as such. The plasma levels found after injecting 4·5 or 9 g of this ester per kg in one single subcutaneous injection are shown in Fig. 2, and Table 2 contains the concentrations of PNP found in the plasma, tumour, liver and brain of male Wistar rats with the mammary tumour injected with a single dose of 4·5–18 g of PNP-butyrate per kg. It is clear that a single injection of even 12 g does not guarantee the desired level of PNP in the tumour for more than 3 hr, and so a following series of rats were injected with multiple doses of PNP-butyrate either simultaneously at different sites or at different times. The results, which are

TABLE 2. THE *p*-NITROPHENOL (PNP) CONCENTRATION IN THE PLASMA, TUMOUR, LIVER, AND BRAIN OF WISTAR RATS WITH A MAMMARY TUMOUR AFTER ONE SUBCUTANEOUS INJECTION OF PNP BUTYRATE

| Dose (g/kg) | Time after injection (hr) | Concentration (M × $10^4$) of PNP in: plasma | tumour | liver | brain |
|---|---|---|---|---|---|
| 4·5 | 1 | 4·4 | | 5·2 | 1·4 |
| | 3 | 2·4 | | 2·4 | 0 |
| | 3 | 1·9 | 1·9 | | |
| | 5 | 2·6 | | 1·5 | |
| | 24 | | | 0·7 | 0·7 |
| 6 | 3 | 2·2 | 2·2 | 2·8 | |
| | 3 | 2·2 | | 1·9 | |
| 9 | 3 | 4·3 | | 3·0 | |
| | 5 | 2·0 | 1·0 | | |
| | 17 | 1·1 | 1·8 | | |
| | 24 | 2·1 | 1·1 | | |
| | 24 | 1·1 | 0·9 | | |
| 12 | 1 | 2·4 | 1·7 | | |
| | 3 | 2·5 | 3·0 | | |
| | 3 | 2·3 | 0·8 | | |
| | 3 | 1·9 | 1·3 | | |
| | 3 | 2·3 | 2·9 | | |
| 18* | 5 | 3·3 | | 0·9 | |

* This dose was very poorly tolerated.

summarized in Table 3, show that even these multiple injections were unable to provide the desired level of PNP in the tumour, although the amounts given were often toxic for the rat.

As the mammary tumour has a high lipid content it was hoped that compounds with a higher lipid solubility than DNP or PNP would reach higher concentrations in tumour tissue than these two compounds. Preliminary experiments with DNCa showed that the required concentration of this compound in the plasma could be obtained by injecting 15 mg/kg intravenously (see Fig. 2). However, even slightly higher doses of DNCa in a bicarbonate solution or substantially higher doses in arachis oil failed to provide the desired levels in the tumour (see Table 4).

The other uncoupling agent with a high lipid solubility which we studied, DNT, proved to be eliminated very slowly; a dose of 20 mg/kg (in 0·21 per cent aq. $NaHCO_3$

or in arachis oil) was well tolerated by adult rats, but when this dose was followed by two doses of 10 mg/kg (in arachis oil) 4 and 3 hr later, all three rats treated in this way died ½ hr after the last injection. It was concluded on the basis of these experiments that a dose of 25 mg/kg per 24 hr probably constituted the maximum dose which would be sustained well by our rats.

TABLE 3. THE *p*-NITROPHENOL (PNP) CONCENTRATIONS IN THE PLASMA, TUMOUR, LIVER AND BRAIN OF WISTAR RATS WITH A MAMMARY TUMOUR AFTER MULTIPLE INJECTIONS OF PNP BUTYRATE HAD BEEN GIVEN EITHER AT THE STATED INTERVALS OR SIMULTANEOUSLY AT DIFFERENT SITES

(Unless stated otherwise all injections were given subcutaneously.)

| Mode of injection | Time (hr) after first injection | Concentration ($M \times 10^4$) of PNP in: plasma | tumour | liver | brain | Remarks |
|---|---|---|---|---|---|---|
| 4·5 g/kg, divided over 3 sites | | | | | | † 30 min after injection |
| 4·5 g/kg, divided over 2 sites | 3 | 3·4 | | 4·6 | 0·7 | |
| | 3 | 2·3 | 2·4 | | | |
| 1·5 g/kg, intraper. +3 g/kg subcut. | 3 | 4·7 | | 5·4 | 0·1 | † 40 min after injection |
| 4·5 g/kg, repeated after 12 hr | 24 | 1·5 | 1·1 | | | |
| 9 g/kg, after 3hr 6 g/kg | 5 | 2·8 | | | | |
| | 5 | 2·3 | | | | |
| 9 g/kg, repeated after 3 hr | 5 | 3·7 | | | | |
| | 5 | 4·2 | | | | |
| | 5 | 5·7 | | | | Rat in poor condition |
| | — | — | — | — | — | † 4½ hr after 1st injection |
| | — | — | — | — | — | † 5 hr after 1st injection |
| 12 g/kg, repeated after 3 hr | 5 | 7·9 | | | | Both animals were in poor condition at time of killing |
| | 5 | 9·2 | | | | |

† Died.

A small series of normal rats and rats with the mammary tumour received daily subcutaneous injections of 10–25 mg of DNT in arachis oil (one rat received a dose of 30 mg) per kg, and the animals were killed at intervals of 1–24 hr after the last injection for the determination of the DNT concentration in the plasma and in the tumour. Two animals died spontaneously 1 and 2 hr after their last injection. The results, summarized in Table 5, indicate that even these relatively high doses were unable to provide a concentration in the tumour equalling or exceeding the level ($4·5 \times 10^{-4}$ M) required effectively to uncouple oxidative phosphorylation.

## DISCUSSION

It is evident from these results that *in vitro* all four uncoupling agents studied are capable of increasing the rate of aerobic glycolysis in the Ehrlich ascites carcinoma to that of the anaerobic glycolysis, i.e. that they can abolish the Pasteur effect completely. However, our efforts to introduce the uncoupling agents in the tumour *in vivo* in the

TABLE 4. THE DINITROCARVACROL (DNCa) CONCENTRATION IN THE PLASMA AND TUMOUR OF WISTAR RATS WITH A MAMMARY TUMOUR AFTER ONE SUBCUTANEOUS (S.C.) OR INTRAPERITONEAL (I.P.) INJECTION

(DNCa was dissolved either in 0·21 per cent (w/v) aq.$NaHCO_3$, "Bic", or in arachis oil, "Oil".)

| Dose (mg/kg) and route | Vehicle | Time after injection (hr) | Concentration ($M \times 10^4$) of DNCa: in plasma | in tumour | Remarks |
|---|---|---|---|---|---|
| 30 i.p. | Bic | 0·75 | 4·0 | | |
| 30 s.c. | Bic | 2 | 2·8 | | |
| 30 s.c. | Bic | 2 | 1·5 | 0·6 | |
| 30 i.p. | Bic | 2 | 5·5 | | |
| 100 s.c. | Oil | 1 | 7·0 | | Four other rats thus treated died after 40 min (2×), 1 hr and 2 hr respectively |
| 100 s.c. | Oil | 3 | 4·2 | 1·8 | |
| 80 s.c. | Oil | 4 | 4·2 | | Two other rats thus treated died after 14 and 24 hr, respectively |
| 70 s.c. | Oil | | | | † After 4 hr |
| 60 s.c. | Oil | 24 | 1·6 | | A third rat thus treated died after 1 hr |
| 60 s.c. | Oil | 1 | 3·9 | | |
| 40 s.c. | Oil | 0·5 | | 1·4 | Determined at autopsy, the rat died 30 min after the injection |
| 40 s.c. repeated after 2 hr | Oil | 4* | 2·4 | 1·4 | |

* After first injection.
† Died.

TABLE 5. THE DINITROTHYMOL (DNT) CONCENTRATION IN THE PLASMA AND IN TUMOUR TISSUE AFTER ONE OR MORE SUBCUTANEOUS INJECTIONS OF A SOLUTION OF THIS COMPOUND IN ARACHIS OIL, IN NORMAL RATS AND WISTAR RATS WITH A MAMMARY TUMOUR

| Animal | Dose (mg/kg) 1st day | 2nd day | 3rd day | 4th day | 5th day | Time after last injection (hr) | Concentration ($M \times 10^4$) of DNT: in plasma | in tumour |
|---|---|---|---|---|---|---|---|---|
| Rats with mammary tumour | 25 | | | | | 24 | 1·12 | 0·42 |
| | 25 | 25 | | | | 2† | | 1·80 |
| | 25 | 25 | 25 | | | 2 | 3·75 | 1·70 |
| | 25 | 25 | 25 | | | 1† | | 1·70 |
| Normal rats | 20 | 20 | 20 | | | 3 | 3·0 | |
| | 30 | | | | | 24 | 2·2 | |
| | 25 | 20 | 20 | 20 | | 24 | 2·1 | |
| | 25 | 20 | 20 | 20 | 10 | 4 | 3·6 | |
| | 25 | 20 | 20 | 20 | 20 | 4 | 3·9 | |

† Died.

concentration required for this effect were not successful. In the case of DNP and PNP it was possible to realize this concentration in the plasma, but not in the tumour, without giving doses that were definitely toxic for the whole animal. The toxicities of DNCa and DNT were even greater so that the required concentration could not even be obtained in the plasma without endangering the life of the rat.

Uncoupling agents with a greater affinity for tumour tissue will therefore have to be found before it will be possible to test the hypothesis that such agents may arrest the growth of malignant cells *in vivo*, by depriving them of an important source of energy. In order to obtain an idea of the characteristics which such compounds should possess we compared some physical and biological characteristics of the four uncoupling agents used (see Table 6). This comparison brings out the fact that the activities of all

TABLE 6. COMPARISON OF SOME PHYSICAL AND BIOLOGICAL CHARACTERISTICS OF DINITROPHENOL (DNP), *p*-NITROPHENOL (PNP), DINITROCARVACROL (DNCa) AND DINITROTHYMOL (DNT)

| Characteristic | DNP | PNP | DNCa | DNT |
|---|---|---|---|---|
| (1) p$K$ | 4·0 | 7·0 | 4·4 | 4·0 |
| (2) Solubility in benzene at 20 °C (M) | 0·45 | 0·12 | 1·87 | 3·73 |
| (3) % bound to protein in ascites fluid | 76 | 57 | 95 | 98 |
| (4) Concentration ($M \times 10^4$) required to increase aerobic glycolysis to anaerobic level in | | | | |
| (a) Ringer solution | 0·5 | 1·5 | 0·3 | 0·05 |
| (b) ascites fluid | 3·6 | 4·7 | 4·8 | 4·5 |
| (5) $\frac{(b)-(a)}{(b)} \times 100$ | 86 | 68 | 94 | 98 |
| (6) Elimination from plasma: half clearance time (approx.) | 2 hr | 10 min | 11 hr | 13 hr |
| (7) Concentration required under 4(b) obtainable in plasma | yes | yes | no | no |
| (8) Concentration required under 4(b) obtainable in tumour | no | yes,* but poorly tolerated | no | no |

* Provided PNP is injected as PNP-butyrate.

four compounds in ascites fluid *in vitro* are of the same order of magnitude, whereas their activities differ markedly when tested in the Krebs–Ringer–bicarbonate solution. The high activity exhibited by DNCa and DNT in the latter medium led us to expect a high activity in ascites fluid too, but here their activity is probably reduced by a high affinity to protein. On the other hand, the activity of these two compounds may be increased by their relatively high lipid solubility, favouring absorption by the tumour cells. Assuming that in ascites fluid only that fraction of the uncoupling agent is active, which is not protein bound, the concentration of the inactive fraction in ascites fluid can be found by subtracting the concentration uncoupling oxidative phosphorylation in Krebs–Ringer–bicarbonate solution from the corresponding concentration in ascites fluid. This difference expressed as a percentage of the concentration required in ascites fluid agrees reasonably well with the percentage bound to protein as determined more directly, viz. by ultrafiltration of ascites fluid to which the uncoupling compound had been added.

It is interesting to note the negative correlation between the affinity to protein and the speed of elimination from the plasma expressed as the half clearance.

On the basis of these admittedly limited observations we should like to draw the tentative conclusion that the uncoupling agents to be applied parenterally against malignant cells should preferably have a minimum affinity for protein, and need not have a higher lipid solubility than PNP in order to reach the tumour from the blood stream in sufficient quantity. The rapid elimination from the plasma exhibited by the latter compound may be due partly to diffusion into the extracellular tissue fluid or into the cells, and as such may be termed desirable. In so far as it is accounted for by elimination in the urine it is of course a disadvantage, which may be met by the use of esters or other kinds of depot preparation, as exemplified by the results obtained with PNP-butyrate.

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