

CI. TUMOUR METABOLISM IN THE PRESENCE OF ANTI-CARCINOGENIC SUBSTANCES.

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THE discovery by Warburg of the characteristic glycolytic metabolism of malignant tumours has been amply confirmed. Nevertheless, little evidence has been found supporting his claim [Warburg, 1930] that the origin of tumours may be referred to the development of a non-oxidative type of metabolism in tissues partially asphyxiated by morbid processes. Orr [1934; 1935] showed that interference with the local circulation accelerated carcinogenesis due to tar and dibenzanthracene and suggested that Warburg's hypothesis might explain such results. It occurred to us that the efficacy of the anti-carcinogenic substances of Berenblum [1929; 1935] might be due to some ability to inhibit glycolysis in cells which were developing the tumour type of metabolism.

Of these anti-carcinogenic substances, mustard gas ($\beta\beta'$ -dichlorodiethylsulphide) has been most fully investigated [Berenblum, 1929; 1931]. A number of chemically related compounds have been similarly tested on tarred mice, as well as certain irritants which, while chemically dissimilar, produce similar anatomical changes on application to mouse skin [Berenblum, 1935]. Of the former group, the irritant members inhibited tumour formation and the others did not, while of the latter group only cantharidin had an effect comparable with that of mustard gas. We have now examined the effect of certain of these substances on tissue metabolism.

METHODS.

The chief difficulty in examining the metabolism of tissues by the manometric method in presence of mustard gas depends on the instability of this compound. At 37° in slightly alkaline solution (bicarbonate Ringer) mustard gas is rapidly hydrolysed with liberation of HCl, and the resultant p_H change may be greater than is permissible in metabolism experiments. This behaviour of mustard gas, as well as its low solubility in water, precluded the use of the usual tissue slice technique. Mustard gas however has a high lipid-water partition coefficient, and we hoped by adding it directly to the tissue to impregnate the tissue lipoids with it and thereby to delay its hydrolysis. It was found that if a drop (roughly 25 mg.) of mustard gas were distributed through 4-5 g. of tissue mince by further mincing with fine scissors, the mustard gas incorporated in the mince had then only a very slight effect on the p_H of the bicarbonate Ringer. For this reason minced tissue was employed in the experiments here described. Manometer readings were begun about 30 min. after the addition of mustard gas.

In our experience the metabolisms of minced and sliced tumour (JRS) tissue differ only quantitatively. Table I gives the mean values obtained for the

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Table I. *Metabolism of minced and sliced tumour tissue.*

		Q_{O_2}	$Q_{CO_2}^{O_2}$	$Q_{CO_2}^{N_2}$
Mince	Mean of 20 experiments	3.7	9.8	—
Mince	Mean of 3 experiments	3.4	9.1	14.5
Slices	Mean of 5 experiments	8.6	23.2	36.3*

* Mean of 2 experiments only.

respiration (Q_{O_2}), the aerobic glycolysis ($Q_{CO_2}^{O_2}$) and the anaerobic glycolysis ($Q_{CO_2}^{N_2}$) of Jensen sarcoma mince and slices. They refer to periods of 40 min. duration at 37°. The figures for the metabolism of slices accord with those of Crabtree [1934], who showed further that the respiration of JRS in phosphate Ringer is 20–30% lower than that in bicarbonate Ringer. Although the metabolism of the mince is considerably lower than that of the slices, the ratio $Q_{O_2} : Q_{CO_2}^{O_2} : Q_{CO_2}^{N_2}$ is almost the same, *i.e.* 1:2.7:4.3 for the mince and 1:2.7:4.2 for the slices. The mechanical injury to the tissue in mincing has therefore depressed proportionately the oxidative and glycolytic metabolisms. Many workers have found that freezing and thawing destroys the glycolytic power of tumour tissue, and similarly attempts to prepare glycolytically active cell-free extracts resembling the active muscle extracts of Meyerhof have consistently failed. These facts, together with the figures for mince and slice metabolism given above, suggest that glycolysis and respiration in tumour tissue are equally dependent upon the integrity of the living cell. The measured metabolism of tumour mince is therefore probably confined to the intact cells, and the relation between the respiratory and glycolytic phenomena in mince is presumably no less normal than in slices.

The Jensen sarcoma tissue was taken from rats immediately after the animals had been killed by a blow on the head. Only peripheral tissue, as free as possible from necrosis, was selected. The tissue was minced with fine curved scissors and the metabolism measured according to the principles established by Warburg. Barcroft differential manometers were used, 0.25 ml. of mince being suspended in 4.75 ml. of phosphate saline for respiration measurements and in the same volume of bicarbonate Ringer containing 0.2% glucose for the measurement of glycolysis. In the latter case the compensating vessel of the manometer contained 0.25 ml. of the same mince in 4.75 ml. of bicarbonate Ringer without glucose. The phosphate Ringer was made up from isotonic solutions of NaCl, KCl, $CaCl_2$, phosphate (p_H 7.4) and 10% glucose in the proportions 50:1:1:20:1.5; the bicarbonate Ringer consisted of isotonic solutions of NaCl, KCl, $CaCl_2$, $NaHCO_3$ in the proportions 50:1.2:1.2:10. The manometers were shaken in a thermostat at 37° and were filled three times, after evacuation at the pump, with O_2 , $O_2 + 5\% CO_2$ or $N_2 + 5\% CO_2$.

$\beta\beta'$ -Dichlorodiethylsulphone, ethylene-bis- β -chloroethylsulphide and cantharidin are also hydrolysed in bicarbonate Ringer at 37°. The sulphone and ethylene compound form hydroxy-derivatives with liberation of free HCl, and the cantharidin is converted into cantharidic acid, of which it is an anhydride (formula, Table IV). The rate of acid production from these substances under the conditions of manometric metabolism experiments was determined. 5 ml. of bicarbonate Ringer were placed in each vessel of the Barcroft apparatus, and shaken with $O_2 + 5\% CO_2$ at 37° until equilibrium was attained. The material under test was then tipped in from a Keilin tube as usual and the course of hydrolysis followed by observation of the CO_2 displacement from the bicarbonate. Fig. 1 shows that the sulphoxide is not hydrolysed, and that the rates of liberation of acid from the sulphone, ethylene-bis-chloroethylsulphide and

cantharidin are mutually similar but less than from mustard gas. Fig. 2 however suggests that the rate of acid liberation from ethylene-bis-chloroethylsulphide and from cantharidin is limited by the low solubility of the compounds since smaller amounts are hydrolysed at greater speeds relative to the sulphone.

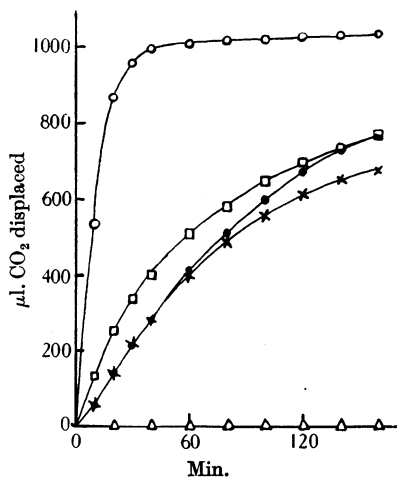


Fig. 1.

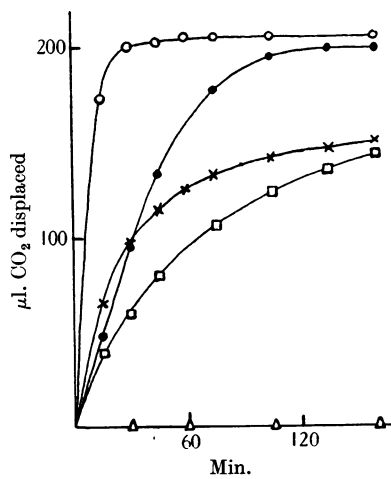


Fig. 2.

Fig. 1. Hydrolysis of mustard gas *etc.* in 5 ml. bicarbonate Ringer at 37°.

- Mustard gas, 4.1 mg. △ $\beta\beta'$ -Dichlorodiethylsulphoxide, 5.2 mg.
 □ $\beta\beta'$ -Dichlorodiethylsulphone, 4.7 mg. × Ethylene-bis- β -chloroethylsulphide, 4.8 mg.
 • Cantharidin, 4.7 mg.

Fig. 2. As Fig. 1 but 1.0 mg. of each substance.

Since these substances are solid at ordinary temperatures it was not desirable to apply them to the mince as in the case of mustard gas. They were added in the form of 5 mg. of a fine powder directly to the medium in the Barcroft vessels in both sides of the manometer.

At the end of many of the experiments the media were transferred in syringes to a glass electrode apparatus of the Stadie type in which the p_H values were measured. The small p_H changes were insufficient to play any part in the effects observed; this was checked in the case of cantharidin by special experiments. The chief danger of acid production, of course, lies in the measurement of glycolysis, for accuracy in this measurement depends on the precise balancing of CO_2 displacement from this cause on the two sides of the manometer. Therefore in a few experiments the manometric measurement of glycolysis was confirmed by chemical estimation of the lactic acid formed, using the method of Friedemann, Cotonio and Shaffer [1927].

RESULTS.

The respiration (O_2 absorption) and glycolysis (CO_2 displacement) of 0.25 mg. of minced tumour tissue with and without impregnation by mustard gas are shown in Table II. The marked inhibition of glycolysis rather than respiration was confirmed in respect of aerobic glycolysis by chemical estimation of the lactic acid formed at the end of the period of manometric observation (Table III). The effects of the other irritants and poisons tested on the metabolism of minced Jensen sarcoma are shown in Table IV.

Table II. *Effect of mustard gas on the metabolism of minced tumour tissue.*

Tissue	Duration of experiment, 40 min.								
	Respiration ($\mu\text{l. O}_2$)		Aerobic glycolysis ($\mu\text{l. CO}_2$)		Anaerobic glycolysis ($\mu\text{l. CO}_2$)		Percentage inhibition by mustard gas		
	Control	Exp.	Control	Exp.	Control	Exp.	Respiration	Aerobic glycolysis	Anaerobic glycolysis
JRS*	99	66	274	54	—	—	33	80	—
JRS	118	83	415	67	—	—	30	84	—
JRS	117	70	338	78	—	—	40	77	—
JRS	131	104	358	115	—	—	21	65	—
JRS	107	94	—	—	305	106	12	—	65
JRS	119	91	—	—	446	129	24	—	71
							Av. 27	76	68
M 63†	67	57	111	29	—	—	15	74	—

* JRS = Jensen rat sarcoma.

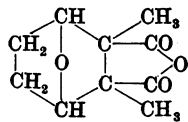
† A transplantable mouse carcinoma kindly supplied by the Imperial Cancer Research Fund Laboratories.

Table III.

Duration of experiment, 1 hour. Figures in brackets represent lactic acid equivalent to CO_2 displaced.

Respiration		Aerobic glycolysis				Percentage inhibition		
		Manometric ($\mu\text{l. CO}_2$)		Chemical (mg. lactic acid)		Glycolysis		
Control	Exp.	Control	Exp.	Control	Exp.	Respiration	Manom.	Chemical
163	150	538 (2.15)	172 (0.69)	1.98	0.58	8	68	71
166	135	369 (1.48)	150 (0.60)	1.48	0.54	19	59	61

Table IV.

Compound	Structure	Respiration ($\mu\text{l. O}_2$)		Aerobic glycolysis ($\mu\text{l. CO}_2$)		Percentage inhibition	
		Control	Exp.	Control	Exp.	Respiration	Glycolysis
Mustard gas	$\text{S}(\text{CH}_2 \cdot \text{CH}_2\text{Cl})_2$	—	—	—	—	27*	76*
Thiodiglycol	$\text{S}(\text{CH}_2 \cdot \text{CH}_2\text{OH})_2$	171	153	490	510	11	— 4
		112	103	318	266	8	16
$\beta\beta'$ -Dichlorodiethyl- sulphoxide	$\text{OS}(\text{CH}_2 \cdot \text{CH}_2\text{Cl})_2$	112	80	318	273	29	14
		130	102	372	253	22	32
$\beta\beta'$ -Dichlorodiethyl- sulphone	$\text{O}_2\text{S}(\text{CH}_2 \cdot \text{CH}_2\text{Cl})_2$	121	61	399	68	50	83
		130	86	372	47	34	87
		88	42	268	41	52	85
Ethylene-bis- β - chloroethylsulphide	$\text{C}_2\text{H}_4(\text{S} \cdot \text{CH}_2 \cdot \text{CH}_2\text{Cl})_2$	136	96	331	260	29	21
		177	150	390	374	15	4
Cantharidin		116	100	368	211	14	43
		131	90	329	115	31	65
		110	73	309	80	34	74
		139	114	324	125	18	61
		132	107	408	211	19	48
Sodium fluoride†		135	132	311	145	2	53
M/500		131	122	329	145	7	56
Acridine 1/1000		116	87	368	416	25	— 13
		135	94	311	381	30	— 22
Croton oil		110	120	309	321	— 9	— 4
		92	134	257	302	— 46	— 18

* Average figures (Table II).

† Ca-free Ringer used in this case.

To ensure that increased acidity of the medium due to hydrolysis was in no way responsible for the inhibitions of glycolysis observed, experiments were carried out in the case of cantharidin in which the acid production from this cause in the experimental vessel was compensated by occasional addition of suitable amounts of alkali (Table V). 0.5 ml. of minced tumour was suspended

Table V.

Time	Additions to	
	Flask A	Flask B
4.00	25 ml. bicarbonate Ringer	25 ml. bicarbonate Ringer
4.07	0.5 ml. JRS mince	0.5 ml. JRS mince
4.09	—	25 mg. cantharidin
4.11	0.5 ml. 10% glucose	0.5 ml. 10% glucose
4.24	0.45 ml. isotonic NaCl	0.45 ml. N/20 NaOH (made isotonic with NaCl)
4.39	0.50 " "	0.50 " "
4.54	0.45 " "	0.45 " "
5.09	0.40 " "	0.40 " "
5.11	Experiment stopped. p_H and lactic acid estimated	
Final p_H	7.30	7.23
Total lactic acid (mg.)	3.72	2.29
Initial lactic acid in mince (mg.)	0.88	0.88
Lactic acid formed (mg.)	2.84	1.41
Inhibition	—	50%

with and without 25 mg. powdered cantharidin, in 25 ml. of glucose-bicarbonate Ringer in 100 ml. conical flasks through which $O_2 + 5\% CO_2$ was passed. The flasks were shaken in the thermostat at 37° . The amount of alkali required was calculated from Fig. 1. Lactic acid formation was determined chemically. In three such experiments the inhibition of aerobic glycolysis was 50, 47 and 50% respectively.

NaF in $M/500$ concentration had only a slight effect on the respiration of JRS mince, but diminished the glycolysis to about half its normal magnitude. Acriflavine (1/1000) had a marked inhibiting action on the respiration whilst increasing the aerobic glycolysis by 10–20%. Croton oil, which is a powerful skin irritant, was unique in that it appeared to stimulate both respiration and glycolysis.

DISCUSSION.

Of the compounds examined in the foregoing section, sodium fluoride and acriflavine have not been tested for anticarcinogenic properties. They were investigated in order to satisfy us that tissue mince under their influence behaved in a manner comparable with that of slices. The results were satisfactory in that NaF exerted a selective action on glycolysis similar to that reported by Crabtree and Cramer [1934] for slices, whilst the behaviour of mince in the presence of acriflavine did not differ from that reported by Mellanby [1933] in the case of slices.

Inhibition of glycolysis to a much greater extent than of respiration was observed with mustard gas, dichlorodiethylsulphone and cantharidin, but not with thiodiglycol, dichlorodiethylsulphoxide or croton oil. The first three are known to inhibit the induction of tar tumours in mice; the others do not produce this effect. This suggested that the capacity for inhibiting glycolysis much more markedly than respiration might be a general characteristic of anticarcinogenic compounds. The behaviour of ethylene-bis- β -chloroethylsulphide, however, does not accord with such a view. This compound is quite as potent

as mustard gas in preventing tar tumours in mice, but its inhibiting effect on glycolysis was rather less than that on respiration. It may, however, be pointed out that the conditions obtaining when it acts in the one case on mouse's skin and in the other on tumour mince are widely different. If the ultimate agent in anti-carcinogenesis were a slowly formed reaction product of ethylene-bis- β -chloroethylsulphide, then the absence of any effect during the short period of the metabolism experiments is understandable.

In a few experiments the effects of mustard gas and of cantharidin on the metabolism of rabbit brain and chick embryo, tissues with a characteristically high anaerobic glycolysis, were examined. In each case the inhibition of anaerobic glycolysis was of the same order as that found for Jensen sarcoma tissue. The respiration of these tissues, however, seemed more sensitive to the irritants than was that of tumour tissue, and this was especially true of embryo, in which the percentage inhibition of respiration was about the same as that of glycolysis.

The depression of respiration of Jensen sarcoma by mustard gas *etc.* increased progressively during the course of an experiment, whilst a simultaneous increase in the inhibition of glycolysis was less marked. The major effect was at first on glycolysis, but the tissue gradually approached a condition in which respiration and glycolysis were almost equally depressed. It is difficult to assess the significance of this. Crabtree and Cramer [1934] suggested that the inhibition of respiration by iodoacetate is an indirect effect accompanying the diminished glycolysis, and the same may hold for the effect with which we are here concerned. On the other hand, the inhibition of glycolysis by mustard gas observed in the later stages of an experiment may be smaller than the true effect of the substance, on account of the considerable decrease in glycolysis of the control tissue, which is largely due, we suppose, to the accumulation of lactic acid and other metabolites.

During the progress of this investigation Jány and Sellei [1935] published a report on the effects of a number of "poison gases" on the metabolism of *B. coli*. It was found that mustard gas inhibited glycolysis by *B. coli* (aerobic glycolysis by 64 %) but that respiration was more than doubled. Similarly the respiration of Ehrlich rat carcinoma was stimulated by 164 % and the aerobic glycolysis inhibited by 94 %. Whilst this inhibition of glycolysis resembles our results with Jensen sarcoma, we have never seen any stimulation of respiration in the latter tissue. Jány and Sellei measured both respiration and glycolysis in bicarbonate Ringer, and appear to have been unaware of the rapidity of hydrolysis of mustard gas in this medium; this may have introduced errors into their results.

SUMMARY.

$\beta\beta'$ -Dichlorodiethylsulphide (mustard gas), $\beta\beta'$ -dichlorodiethylsulphone and cantharidin, which inhibit the induction *in vivo* of tar tumours, reduce the glycolysis of minced Jensen sarcoma tissue *in vitro* more than its respiration.

$\beta\beta'$ -Dichlorodiethylsulphoxide, thiodiglycol and croton oil, which have no inhibiting effect on carcinogenesis, do not reduce glycolysis more than respiration.

Ethylene-bis- β -chloroethylsulphide, which inhibits the induction of tumours, has no selective action on glycolysis.

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