THE INFLUENCE OF UREA DERIVATIVES ON THE METABOLISM OF NORMAL AND TUMOR TISSUES

By

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For her generous assistance throughout this investigation, I would like to acknowledge a debt of gratitude to my wife. I wish also to thank Dr. John C. Krantz, Jr., and the International Cancer Research Foundation for providing the opportunity and necessary facilities, and Miss Dorothy Kibler and Dr. Sylvan E. Forman for chemical syntheses.

W.C.G.

TABLE OF CONTENTS

LIST OF TABLES

FIGURES

INTRODUCTION

Object

A study of the influence of various derivatives of urea on cellular metabolism has been undertaken with a twofold purpose:

1- to compare the reactions of various normal and tumor tissues.

2- to determine the mode and site of action of these compounds.

Biological oxidations

A discussion of the history and voluminous literature of Biological oxidations is far beyond the scope of this paper. Extensive treatment of this subject is presented in the reviews by Dixon (1938), Oppenheimer and Stern (1939), Szent-Gyorgyi (1939), Elvehjem and Wilson (1939), and Elliott (1941), and many others. It will thus suffice to outline a few of the systems examined in the course of study.

1. Respiration. True respiration has its site in the cell and is concerned with the oxidation of foodstuffs with the controlled release of energy. Cellular enzymes activate the ultimate union of the hydrogen of the substrate with oxygen. It is from this combination of hydrogen and oxygen that the vital energy of life is drawn.

$$2H + \tfrac{1}{2}O_2 = H_2O + \text{Energy}$$

The liberated energy may be employed simply as a source of

heat or as the driving force in one of the numerous body f functions.

The foregoing combination is a result of many secondary reactions dealing mainly with the release and transportation of hydrogen of the foodstuff to oxygen. Typical examples of the types of enzyme systems involved will be described.

2. Dehydrogenase. Hydrogen occurring as a constituent of foodstuffs is relatively inert at body temperature. In order that hydrogen may be released, participation of a dehydrogenating enzyme or dehydrogenase is essential. These substances are known to be protein in nature but their exact mode of "activating" hydrogen for release is as yet unsolved.

The known dehydrogenases are specific as regards their substrates, e.g., lactic acid, succinic acid, glucose dehydrogenases, but act in cooperation with cert in other non-specific cellular enzymes.

3. Coenzymes. It was early discovered in the case of most physiological substrates or foodstuffs that the specific dehydrogenase required the activity of a second enzyme in removing hydrogen from the substrate. The latter substance was termed a co-dehydrogenase, or more commonly, a co-enzyme and is recognized in two forms, Coenzymes I and II. Chemically, I is a di-phosphopyridine nucleotide, and II is a tri-phophopyridine nucleotide. Upon hydrolysis, Coenzyme I yields phosphoric acid, ribose, adenine, and nicotinic acid amide.

When, for example, lactic acid is added to a mixture

of its dehydrogenase and Coenzyme I, pyruvic acid is produced and the nicotinic acid amide of the coenzyme is reduced by addition of hydrogen.

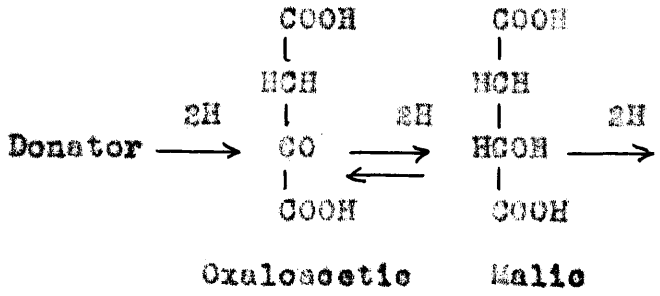
$$2 \text{ Lactic acid} + \text{Coenzyme} \xrightarrow[\text{dehydrogenase}]{\text{Lactic}} 2 \text{ Pyruvic acid} + \text{Coenzyme} \cdot 2H$$

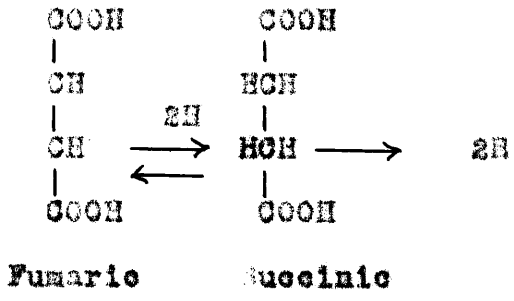
4. Yellow Enzyme. The dehydrogenation or oxidation of the reduced coenzyme is effected through the mediation of Warburg's Yellow Enzyme. This substance, flavoprotein, consists of a high molecular weight protein and a prosthetic group. The latter, called riboflavin, is composed of phosphoric acid, ribose and iso-alloxazine. The 2 atoms of hydrogen are transferred from the nicotinic acid amide of reduced Coenzyme I to the iso-alloxazine ring of the flavoprotein's prosthetic group. In this manner reduced Coenzyme I is reoxidized and the flavoprotein reduced.

$$\text{Coenzyme} \cdot 2H + \text{Flavoprotein} \longrightarrow \text{Coenzyme} + \text{Flavoprotein} \cdot 2H$$

5. Four Carbon Dicarboxylic acids. Another "hydrogen transport" system interposed between the primary foodstuff and oxygen is the succinic acid series of Szent-Gyorgyi (1939). Neglecting momentarily the enzymes involved, the following is an outline of the reactions which occur when labile hydrogen is added to oxaloacetic and fumaric acids. Oxaloacetic acid is reduced to malic by the addition of two atoms of hydrogen. These atoms are then transferred to fumaric acid which becomes succinic on hydrogenation.

By virtue of dehydrogenation, malic acid reverts to the original oxaloacetic acid.





6. Terminal Respiratory Complex. The systems described have been concerned with (a) "activation," (b) removal, and (c) transport of substrate hydrogen. The final stage in hydrogen transfer is effected specifically in animal tissues by succinic dehydrogenase. This enzyme removes 2 atoms of hydrogen from succinic acid, thus oxidizing it to fumaric; and the hydrogen passes to cytochrome which is thereby reduced.

Cytochrome is a hematin containing protein which functions as the specific link between the hydrogen transport systems and oxygen. Iron in oxidized cytochrome is in the ferric condition and is readily reduced by electrons from added hydrogen to the ferrous state. An enzyme, cytochrome oxidase, "atmungsferment" of Warburg, indophenol oxidase, etc., re-

oxidizes the reduced cytochrome in a manner which is not entirely understood. The main function of cytochrome oxidase is apparently its ability to "activate" molecular oxygen, thus rendering it susceptible to union with hydrogen from the reduced cytochrome. The final result is the oxidation of reduced cytochrome and production of water or hydrogen peroxide, the latter being rapidly decomposed by the enzyme, catalase.

7. Example. It must be emphasized that the mechanisms discussed are but theoretical representations and that various combinations of these systems are possible. In addition, for every enzyme mentioned above, at least 10 more have been examined. A possible chain of reactions is presented in Table I merely as an example. Since little is known of the preliminary aerobic breakdown of metabolites, none is shown.

TABLE I

$$\text{Substrate} \cdot 2H + \text{Coenzyme} \xrightarrow[\text{dehydrogenase}]{\text{Specific}} \text{Substrate} + \text{Coenzyme} \cdot 2H$$

$$\text{Coenzyme} \cdot 2H + \text{Oxaloacetic} \xrightarrow[\text{dehydrogenase}]{\text{Malic}} \text{Coenzyme} + \text{Malic}$$

$$\text{Malic} + \text{Flavoprotein} \longrightarrow \text{Oxaloacetic} + \text{Flavoprotein} \cdot 2H$$

$$\text{Flavoprotein} \cdot 2H + \text{Fumaric} \longrightarrow \text{Succinic} + \text{Flavoprotein}$$

$$\text{Succinic} + \text{Cytochrome} \xrightarrow[\text{dehydrogenase}]{\text{Succinic}} \text{Fumaric} + \text{Cytochrome} \cdot 2H$$

$$\text{Cytochrome} \cdot 2H + O_2 \xrightarrow[\text{oxidase}]{\text{Cytochrome}} \text{Cytochrome} + H_2O_2$$

$$2\ H_2O_2 \xrightarrow[\text{catalase}]{} H_2O + O_2$$

EXPERIMENTAL

Measurement of Gas Exchange

By far the greatest portion of results recorded was obtained by the use of the Warburg Respiratory Manometer. Over a thousand individual determinations have been made by this method. Other techniques employed will be described under appropriate sections. The theory and practical details of the Warburg Manometer have been admirably enumerated by Warburg (1926) and Dixon (1934) and will not be dealt with here.

In the determination of oxygen uptake, Ringer's solution of the following composition was added to the reaction vessel:

Substance	Concentration	Parts
NaCl	0.15 M	100
KCl	0.15 M	2
$CaCl_2$	0.15 M	2
Na_2HPO_4	0.066M	10
KH_2PO_4	0.066M	

The phosphate buffer was pH of 7.38 at 38° C. When desired, 2 parts of 10% glucose were added to the above solution. The volume in contact with the respiring tissue was always adjusted to 3.0 cc. regardless of the addition of reagents. The gas phase of the reaction vessel was oxygen.

When anaerobic glycolysis (lactic acid production) was measured, bicarbonate-Ringer's solution of the following composition was added:

Substance	Concentration	Parts
NaCl	0.15 M	96
KCl	0.15 M	2
$CaCl_2$	0.15 M	2
$NaHCO_3$	0.15 M	20
Glucose	10%	2.4

The resulting solution contained bicarbonate in the physiological concentration of 0.025 M. and glucose 0.2% and is in contact with a gas phase consisting of 95% N_2 and 5% CO_2. The volume of solution in contact with tissue was 3.0 cc.

During aerobic work, the central well contained 0.2 cc. of a 20% solution of KOH and a roll of filter paper made from a 1.5 by 3.0 cm. rectangle. In the anaerobic measurements this CO_2 absorption system was omitted.

Addition of a reagent while a determination was in progress was made from the side bulb of the reaction vessel. The reagent was present in the side bulb at 10 times the concentration desired, since the volume of the addition was 0.3cc. and the final volume in the reaction vessel 3.0 cc.; the concentration of the added reagent was thus reduced ten times.

Manometer reaction vessels were suspended in a constant temperature water bath at 38° C. The shaking device permits a swing of 4.5 cm. and a rate of 110 complete oscillations per minute. A preliminary temperature equilibration period of 15 minutes ensued with stop-cocks open before final closure and readings.

Oxygen consumption is expressed in the conventional manner as the Qo_2, cubic millimeters of oxygen absorbed per milligram of dried (105°C.) tissue per hour. Anaerobic gly-

colysis is represented by $Q^{N_2}_{CO_2}$, cubic millimeters of carbon dioxide produced per milligram dried (105°C.) tissue per hour. It is to be stressed that these two symbols, Q_{O_2} and $Q^{N_2}_{CO_2}$, signify rate and not necessarily total consumption or production in the 60 minutes specified by the definitions. In the case of the former, one may measure oxygen uptake for 30 minutes or 10 minutes and express the result as the Q_{O_2} after having multiplied the cubic millimeters of oxygen consumed by 2 or 6 and divided by the weight of the dried tissue.

Materials

Male albino rats, 100 to 200 gms., were used throughout this study. The animal was stunned by a sharp blow on the occipital base, and death by exsanguination followed severing the large cervical vessels. Slices of brain (cerebrum), liver, skeletal muscle (deaphragm), kidney, spleen, and Walker sarcoma #319, 0.3 to 0.4 mm. in thickness, were cut immediately in the conventional manner. The elapsed time between stunning and equilibration of the manometers in the constant temperature bath averaged 25 minutes. Different methods of tissue preparation have been employed in this study and will be described under the appropriate sections.

The tumor used throughout this investigation was the Walker rat sarcoma #319. It was discovered in the peroneum of a female albino rat by Dr. George Walker in 1929, in Baltimore. Since that date it has been carried in rats and tissue culture by Drs. Warren H. Lewis and George O. Gey.

For optimal growth, the sarcoma was transferred at

2 to 3 week intervals from rat to rat. Central necrosis began at about the third week and soon invaded all but a peripheral shell of growing tissue. Samples used in respiratory study were taken from non-necrotic areas.

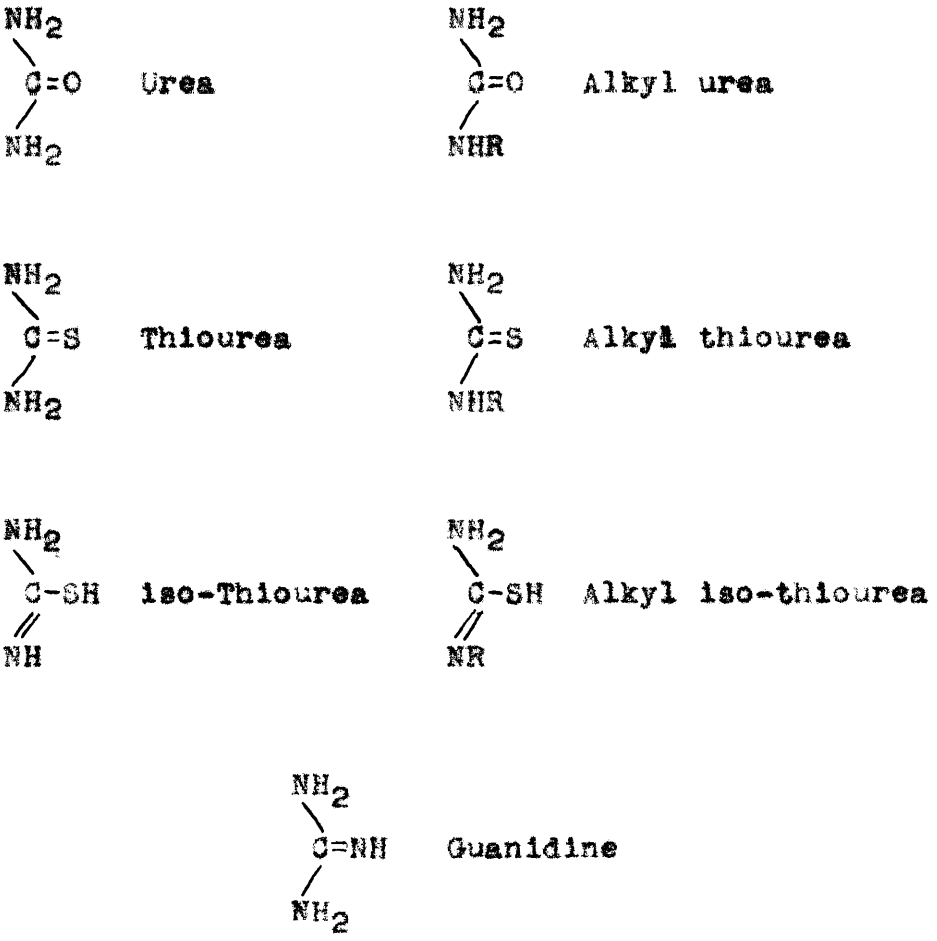
Morphologically, the sarcoma is characterized by a tough, collagenous capsule which successfully confines the neoplastic tissue. Microscopic sections made at intervals revealed the presence of large round and spindle cells with pale cytoplasm. The nuclei were abundantly filled with weakly staining chromatin material and usually contained a large and prominent nucleolus. The tissue is ideally suited to metabolic studies owing to the relative freedom from stroma which thus enhances the "purity" of the cell population.

Certain of the urea derivatives used were obtained from the Eastman Kodak Company of Rochester, New York. The majority were synthesized from nitroureas and the desired amine by Miss Dorothy Kibler and Dr. Sylvan E. Forman of this laboratory. The synthesis is outlined in Table II.

All reagents employed were dissolved in physiological saline (0.15 M). Those compounds subject to decomposition by bacterial or mold contamination (such as metabolites) were kept in a refrigerator and prepared every 3 to 4 days. Unstable compounds, p-phenylenediamine etc, were dissolved immediately before addition to the reaction vessel.

TABLE II

RELATIONSHIP OF THE UREA DERIVATIVES



SYNTHESIS OF THE ALKYL UREA DERIVATIVES

$$NH_2CONH_2 + HNO_3 \longrightarrow NH_2CONH_2 \cdot HNO_3$$

$$NH_2CONH_2 \cdot HNO_3 \xrightarrow{H_2SO_4} NH_2CONH \cdot NO_2$$

$$NH_2CONH \cdot NO_2 + RNH_2 \longrightarrow NH_2CONHR + N_2O + H_2O$$

RESULTS

Derivatives of Urea

All compounds employed in this section bear a close relationship to urea. Thiourea, which contains sulfur in lieu of the ketone oxygen of urea, and guanidine, which has an amino group instead of oxygen, were also used. Alkyl, or acyl, groups with varying numbers of carbon atoms were substituted for a single amino group hydrogen atom, thus creating a related series of derivatives. These compounds are portrayed graphically in Table II.

Oxygen Consumption

Inasmuch as a great deal of the following section will deal with respiration or oxygen consumption in terms of percentage change from an initial normal to a later addition or experimental period, it seems fitting that absolute values for normal oxygen uptake rate (Qo_2) be given.

	Brain	Liver	Muscle	Tumor
Average Qo_2	10.3	9.4	6.3	9.3
No. of Det'n	59	64	52	83

Table III presents the results of adding an homologous series of derivatives, urea, methyl urea, ethyl urea, n-propyl urea, and n-butyl urea to tissue slices. n-Amyl urea was omitted because of the marked decrease in water solubility, which occurs with the transition from the four to the five carbon atom side chain.

In determining the influence of the urea on oxygen con-

sumption, a slice of tissue was allowed to respire in Ringer's solution for 30 minutes. The Qo_2 was taken as normal for that individual slice of tissue. As individual variations are often rather large, this procedure is necessary. The urea derivative was then added from the side bulb and the respiratory rate followed for the subsequent 60 minutes. In order to evaluate the effect of the added agent, the percentage deviation, or change ( ), of Qo_2 for the addition period from

TABLE III

The effect of an homologous series of urea derivatives on oxygen consumption for 60 minutes. Results are expressed as percentage change from controls.

Derivative	M	No. det'n	Brain %	Liver %	Muscle %	Tumor %
Urea	0.2	16	1	0	10	- 7
Methyl urea	0.1	11	- 8	- 7	10	- 4
Ethyl urea	0.1	24	-11	- 5	6	8
Propyl urea	0.1	29	- 8	- 9	2	- 29
Butyl urea	0.1	13	- 68	- 51	- 29	- 66

that for the normal was taken. Since any untreated tissue respiring <u>in vitro</u> will, in the course of 90 minutes, tend to fall off in its rate of oxygen consumption, a control percentage deviation was determined. To learn the true effect of an added agent, the percentage deviation of control is subtracted <u>algebraically</u> from that of the addition. For example, in brain, methyl urea causes a percentage deviation of -17. Since untreated brain will have a percentage deviation of -6, hence the true change in brain to be attributed to the agent is -11%.

In Figur 1 the results of Table III are represented as a

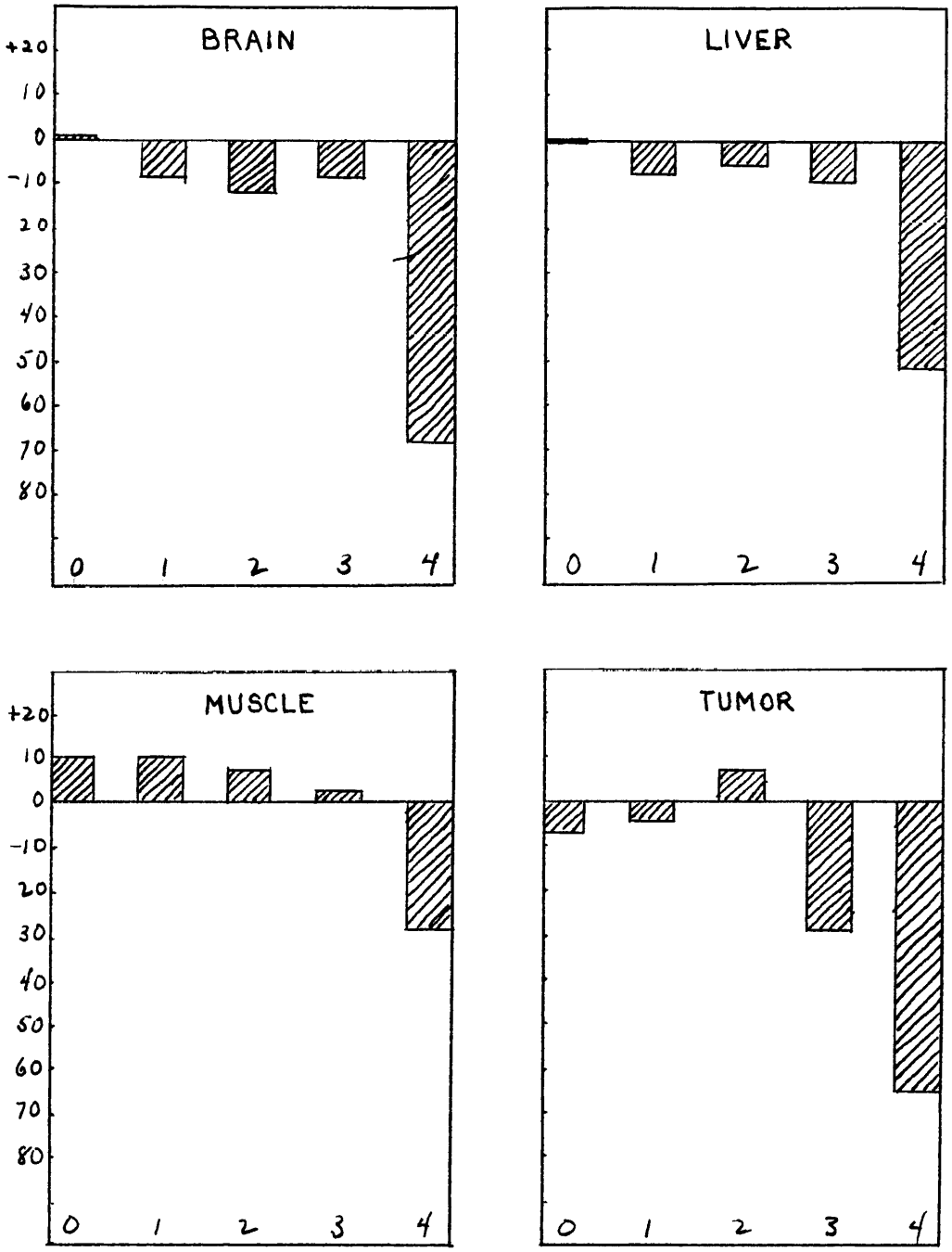


Figure 1

The effect of an homologous series of urea derivatives on Q_{O_2}. Results are expressed in ordinates as percentage change from controls. Abscissae represent the number of carbon atoms in the side chain.

bar graph. It will be noted that brain and liver are rather similar in reaction to all five ureas. The former is, however, more sensitive to the depressant action. The addition of the fourth carbon atom in the form of the n-butyl side chain brings about a marked drop in respiration. In the case of skeletal muscle (diaphragm), urea, methyl, ethyl, and n-propyl derivatives elicit a small increase in rate. Also the depression of rate by n-butyl urea is much less than that in the other tissues. Muscle has the lowest Qo_2 of the four tissues and this fact may partially explain its resistance to agents. The slight stimulation evidenced by the afore mentioned compounds persists only during the first 60 minutes following the addition and subsequent depression ensues.

Tumor does not resemble very closely the normal tissues in its reaction to the five ureas. As seen in Figure 1, there is evidence of a minimum of depression at the two carbon atom side chain, ethyl urea, whereas the normal tissues show a trend of increased depression from urea to n-butyl urea. The drop in oxygen consumption rate with n-butyl is quite in keeping with the others, but that with n-propyl is much larger.

Table IV contains a group of urea derivatives which were studied with the purpose of uncovering any marked specificity in action. Since the solubility of certain compounds, the acyl derivatives, iso-thioureas etc., is relatively low, comparison on an equi-molecular basis was made difficult.

Little difference was observed between the n-alkyl ureas and the corresponding iso-alkyls. The substitution of sulfur

TABLE IV

The effect of certain urea derivatives on oxygen consumption rate for 60 minutes. Results are expressed as percentage change from controls.

Derivative	M	No. of det'n.	Brain %	Liver %	Muscle %	Tumor %
n-Amyl urea	0.01	12	15	4	17	-18
Acetyl urea	0.01	12	- 4	7	7	8
Propionyl urea	0.008	9	19	- 3	5	- 2
Allyl urea	0.1	8	- 17	- 19	-13	-31
iso-Propyl urea	0.1	9	- 12	- 4	- 6	-20
iso-Butyl urea	0.1	4	- 66	- 41	- 22	--
"	0.05	5	- 26	- 11	- 6	-37
iso-Amyl urea	0.01	4	1	- 3	13	- 1
Thiourea	0.1	10	- 10	- 6	1	-10
Methyl thiourea	0.1	8	- 7	- 1	8	-15
Ethyl thiourea	0.1	7	- 7	- 12	14	-10
Acetyl thiourea	0.01	5	- 9	5	21	- 1
Methyl iso-thiourea	0.01	7	11	2	19	12
Benzyl iso-thiourea	0.05	5	- 88	- 30	- 10	--
"	0.01	8	- 62	0	1	- 43
"	0.001	2	--	--	--	- 10
Guanidine	0.1	8	- 67	-24	- 5	-37
"	0.05	5	- 36	- 27	17	- 35
"	0.01	8	- 48	- 14	14	2
"	0.005	3	- 36	- 15	--	0

for oxygen in the thioureas brought about no increase in depressant action. The iso-thioureas, methyl and benzyl, were less soluble than the alkyl ureas, but a comparison of these two is possible. Methyl iso-thiourea produced a small increase in Qo_2, whereas benzyl iso-thiourea at the same molar strength all but abolished the respiration in brain with somewhat less effect on tumor and no effect on liver or muscle. The striking difference in depressant action is caused by substituting a benzyl for a methyl group. The marked specificity of depression of brain and tumor is interesting and may be linked with the fermentative ability shared by these tissues.

Anaerobic glycolysis

Many tissues have the ability to utilize metabolites in two ways: (1) oxidation, or a removal of hydrogen by the dehydrogenases and transfer to molecular oxygen; and (2) a series of intermediates and ultimately lactic acid. It is this latter process that is discussed in this section.

In order to measure glycolysis, advantage is taken of the acidity of the end product, i.e., lactic acid. As lactic acid is produced by the cells in an anaerobic environment, a corresponding amount of carbon dioxide is released from the $NaHCO_3$ of the medium, thus the pressure is increased in the manometer. Results are expressed in terms of cubic millimeters of carbon dioxide produced per 60 minutes per milligram dried tissue, $Q^{N_2}_{CO_2}$.

The influence of several ureas on the glycolysis of four tissues is presented in Table V. These results contrast

markedly with those in terms of oxygen consumption. Whereas the latter was largely depressed in both normal and tumor cells, glycolysis of the normals, at least, is unaffected. Tumor with its high rate of glycolysis is more sensitive to the ureas, particularly n-propyl and n-butyl; however the Q_{O_2} is more sensitive than the $Q^{N_2}_{CO_2}$.

Owing to the anaerobic conditions, the viability of the cells diminishes rapidly; for this reason, measurements must

TABLE V

Influence of urea derivatives on anaerobic glycolysis. Figures represent $Q^{N_2}_{CO_2}$ in control and addition periods.

Agent (M)		Brain		Liver		Tumor	
		Con.	Add.	Con.	Add.	Con.	Add.
Methyl urea	0.1	9.6	13.7	7.5	5.7	30.0	26.7
Ethyl urea	0.1	10.4	11.4	5.5	5.1	34.3	23.9
Propyl urea	0.1	9.0	9.5	3.5	4.2	26.0	19.4
Butyl urea	0.1	10.4	15.4	5.5	7.1	26.3	15.8
Amyl urea	0.01	9.6	13.4	7.5	11.7	28.7	29.2
Guanidine	0.01	-	-	-	-	25.3	26.9

be confined to some 20 to 30 minutes. Hence an initial normal period cannot be obtained and the customary percentage change or deviation is not used. This type of metabolic measurement is, therefore, not as precise as is the preceding one; but it throws some light on the lactic acid production of the tissues while under the influence of added reagents.

Methylene Blue Reduction

In order to localize the site of the depressant action of urea derivatives on tissue metabolism, recourse was made

to the apparatus of Thunberg (1920). By this method, the activity of certain dehydrogenation enzymes may be measured using a chromatic criterion.

There exist in all tissue, enzymes which are capable of activating and removing the labile hydrogen atoms of many cell metabolites. The hydrogen removed is transferred ultimately to a compound called a "hydrogen acceptor,"usually oxygen. The path of hydrogen from the primary metabolite to its final union with oxygen is often a long and complex one, but may be omitted in this present discussion. Methylene blue is an active and reducible dye which can act as an artificial "hydrogen acceptor." Upon reduction by the added hydrogen, the characteristic blue fades and the compound assumes the colorless leuco form. The rate of reduction of the dye may, therefore, be used as an index of dehydrogenase action (Quastel and Whetham, 1924). Since leuco methylene blue is readily reoxidized by atmospheric oxygen to the colored form, anaerobic conditions are maintained.

Finely minced tissue suspended in Ringer-phosphate solution is placed in a specially constructed Thunberg tube. This vessel is a 30 cc. test tube with ground-glass stopper and side bulb. The system can be evacuated by means of a high efficiency pump and sealed by turning the stopper. The filled tubes are maintained at constant temperature (38°) until thermal equilibrium is established. Methylene blue is then added to the tissue suspension from the isolated side bulb. The time required for complete disappearance of the blue color thus furnishes an index of dehydrogenation rate.

Brain, liver, and tumor were ground in a mortar and placed in Thunberg tubes with members of the homologous series of urea derivatives. The results are presented in Table VI and Figure 2 in terms of time (minutes) required to complete decolorization of methylene blue. A control determination is included to afford a basis of comparison. For example, in brain with no added urea derivative, 24 minutes sufficed for removal of the blue color. When ethyl urea was present, 41

TABLE VI

Influence of urea derivatives on anaerobic dehydrogenation. (Methylene blue reduction time.)

Agent	(M)	Brain min.	Liver min.	Tumor min.
None		24	14.5	43
Urea	0.2	28	14.5	45
Methyl	0.1	33	17.5	65
Ethyl	0.1	41	17.5	71
Propyl	0.1	116	18.0	145
Butyl	0.1	> 150	20.0	> 150

minutes were required, indicating an inhibition of dehydrogenation.

It will be noted that in all three tissues, the influence of these compounds is one of depression and that this action increases with the length of the alkyl side chain. Brain and tumor were affected in very much the same manner, whereas liver proved to be more resistant.

The main facts to be derived from this experimental series are confined to a comparison of the influences of the derivatives on a single tissue. Since we are dealing with a finely ground cell suspension in dilution instead of the more intact

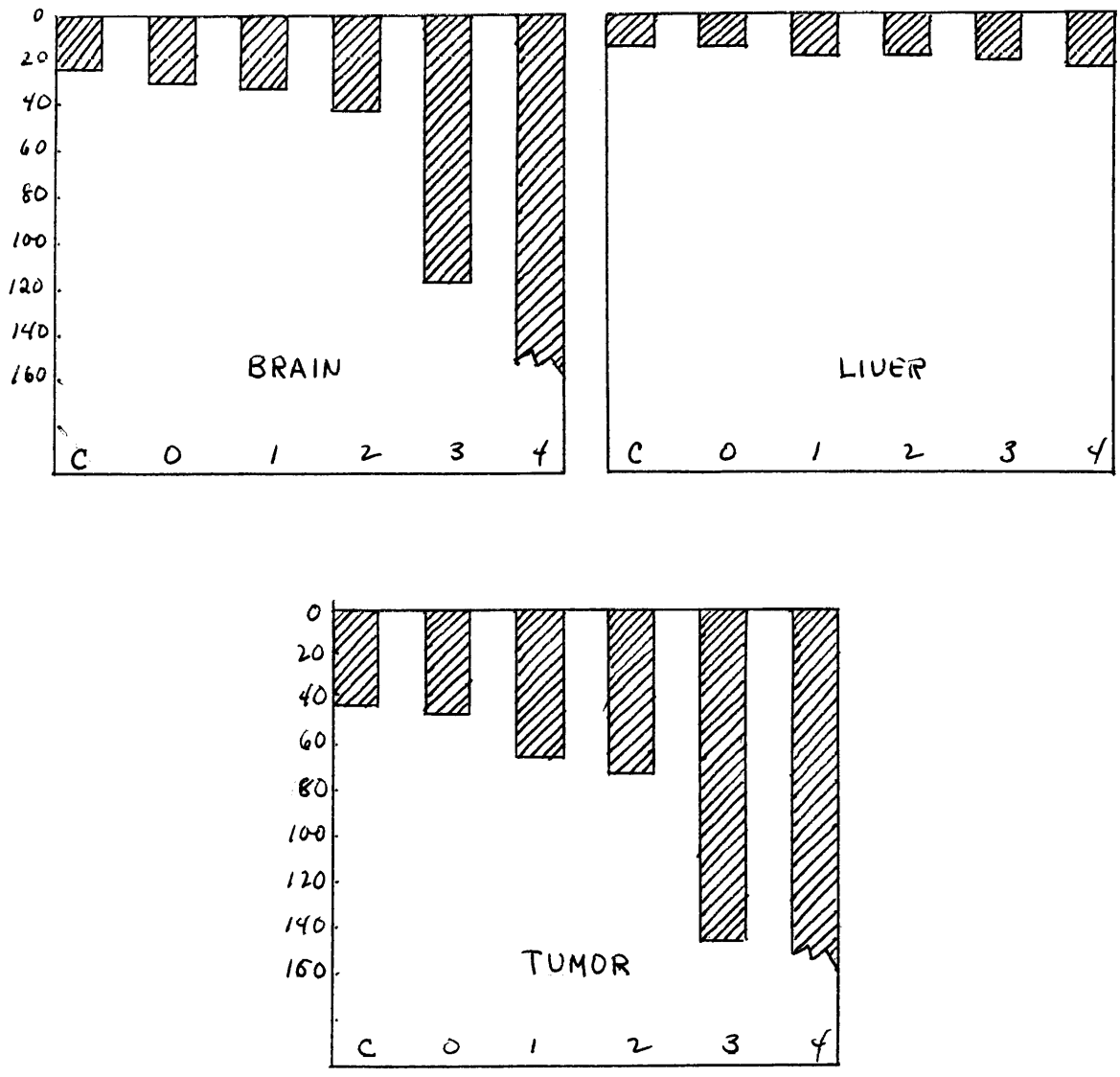


Figure 2

The effect of added urea derivatives on methylene blue reduction time. Abscissa, C is the control and 0,1,2,3,4, represent the number of carbon atoms substituted in the side chain. Ordinate, time in minutes.

and normal tissue slices, comparison between two tissues is at best approximate. Each urea was assayed on an aliquot of a single tissue brei and hence evaluation of the effect of various ureas on one tissue is quite valid.

n-Butyl urea as sample urea derivative

In order that the modus operandi of the derivatives of urea might be studied more intensively, n-butyl urea, $NH_2CONHC_4H_9$, was selected as the example to be investigated.

Table VII

Effect of varying the concentration of n-butyl urea on oxygen consumption rate.

n-Butyl urea (M)	Brain Qo_2	Brain % change	Liver Qo_2	Liver % change	Tumor Qo_2	Tumor % change
0.10	3.3	-71	4.6	-37	2.1	-72
0.08	4.6	-64	5.2	-30	3.2	-61
0.06	7.5	-38	6.2	-11	4.4	-39
0.04	8.8	-33	6.5	-8	6.1	-31
0.02	12.4	-14	7.2	-8	8.1	-10
0.00	14.8	0	8.7	0	10.5	0

it is an extremely active member of the series and qualitatively appears typical in action.

1. Concentration variable. Butyl urea in a series of graded molarities was added to respiring tissues, and the percentage change in Qo_2 over that of a normal period was measured. These data are shown numerically in Table VII and graphically in Figure 3. As would be anticipated, the extent of depression is directly proportionate ti molarity. Brain and sarcoma are similar in their responses to each strength of butyl urea, whereas liver is more resistant to depression than either.

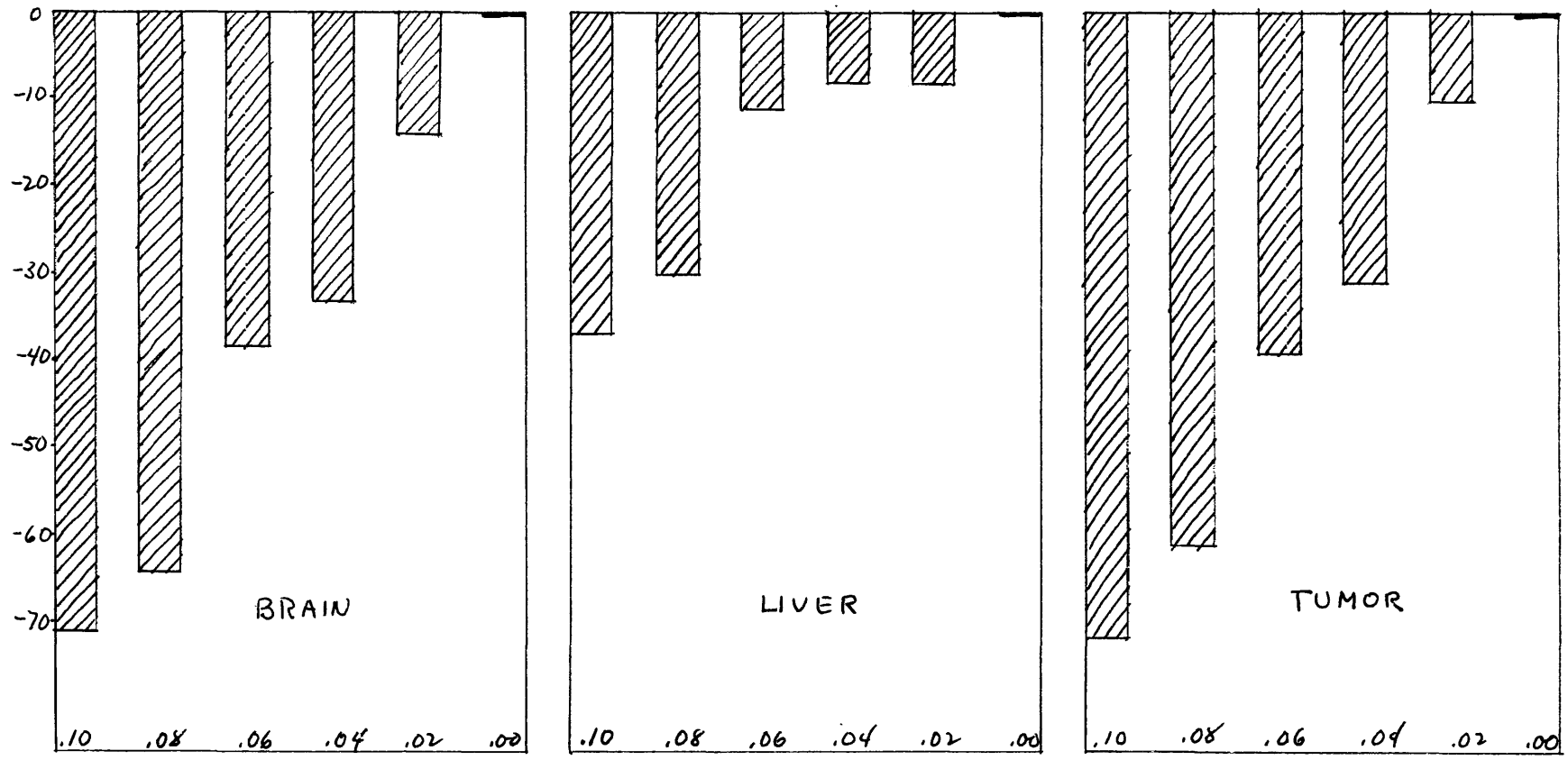


Figure 3

The influence of varying the concentration of n-butyl urea on oxygen consumption. Ordinates represent percentage change from normal to addition period after subtraction of that of zero concentration. Abscissae, molar concentration.

it will be noted that in all tissues a marked falling off in action coincides with passage from 0.08 M to 0.06M. Owing to solubility difficulties, concentrations beyond 0.10M were not employed.

2. Reversal of action. All studies thus far have dealt with the influence of urea derivatives on the metabolism of cells suspended in a medium containing glucose. In other words, all results which have been presented are a measure of the biological oxidation of glucose or one of its break down products, e.g. lactic acid. In attempting to elucidate further the site of the urea action, a number of compounds both physiological and synthetic were substituted for glucose in the medium. While it is generally conceded that glucose and lactic acid are the chief carbohydrate metabolites in vitro and very likely in vivo (Dickens and Greville 1933, Ashford 1933, Sherif and Holmes 1930, Quastel and Wheatley 1932) even though the relative importance of the two is much disputed (Himwich and Fazikas 1935; Jowett and Quastel 1937; Baker, Fazikas and Himwich 1938), certain other substances can be shown to act as substrates. In addition, there exists a class of compounds, both natural and unnatural, which act catalytically on metabolic processes.

The procedure adopted was to measure quantitatively the change in Q_{O_2} produced by the addition of a metabolite or respiratory stimulant to a tissue previously treated with n-butyl urea, usually 0.10M. In some cases, the urea was added after the metabolic agent.

(a) 2,4-Dinitrophenol. That 2,4-dinitrophenol causes an increase in the rate of oxidation and hyperthermy in warm blooded animals is well known, but a satisfactory explanation of these phenomena has not been forthcoming (Oppenheimer and Stern, 1939). Ehrenfest and Ronzoni (1933) have demonstrated an increase in anaerobic glycolysis of normal and tumor tissue slices under the influence of 2,4-dinitrophenol, and suggest that the augmented oxygen uptake is a result of heightened lactic acid concentration caused by the glycolytic stimulation. On the other hand, Dodds and Greville (1933) found an increase in Q_{O_2} of rat kidney which is a tissue devoid of aerobic glycolysis and hence unable to produce lactic acid. Oppenheimer and Stern (loc. cit.) reject the hypothesis that the respiratory stimulation of 2,4-dinitrophenol is based upon its ability to act as an acceptor of hydrogen or to stimulate formation of lactic acid as respiratory metabolite. Their suggestion is that the increased oxygen consumption is rather a "pharmacological effect on the regulatory mechanism of cell respiration."

The addition of 0.0005% 2,4-dinitrophenol to brain slices brings about a marked increase in Q_{O_2}, as will be seen in Table VIII. n-Butyl urea 0.10M completely abolishes the stimulation of respiration by 2,4-dinitrophenol, and Q_{O_2} falls to the characteristic depressed level. Liver responds in a similar manner.

From these results, it would appear that the depressant action of n-butyl urea and the stimulant action of 2,4-dinitrophenol have a similar locus in the chain of respiratory

oxidase) is blue-violet, Bandrowski's Base with its bronze color cannot be the entire product. In his original research, Bandrowski mentions a blue-violet contamination which he was unable to explain. Dr. Donald L. Vivian, of this laboratory, suggests that the blue-violet may well result from semi-quinone formation, similar to the well known hydroquinone-quinone compound, quinhydrone. In the case of Bandrowski's Base, a molecular compound composed of the base formulated above and the reduced form in which the double bonds to the nitrogen atoms have been replaced by single bonds is postulated.

The chemistry of the oxidation product or products of p-phenylene diamine is of interest because of the intensive use which is made of this compound in Biological oxidations. Workers in this field dismiss the subject with the statement that the product of oxidation is the di-imine, $C_6H_4(NH)_2$. Examination of a text-book of Organic Compounds (Whitmore 1937)) shows this compound to be yellow. Obviously, further explanation is in order when one considers that the color of a biologically oxidized solution possesses a definite blue-violet cast.

In Table IX, the results of the addition of neutralized p-phenylene diamine hydrochloride to brain slices are presented. n-Butyl urea, 0.10M, was present in some cases and absent in others, as indicated. Owing to the instability of p-phenylene diamine, the usual preliminary control period was omitted. It will be noted that the Q_{O_2} of brain in a glucose free medium averaged 7.1. Upon the addition of p-phenylene diamine (19 and 25 mg. per 3.0 cc. of medium), the

rate rose to an average value of 22.1 and 23.5, thus demonstrating the enormous oxidative capacity of brain tissue (cytochrome oxidase) for this substance. The presence of n-butyl urea has no appreciable effect on this reaction, whereas the sample treated with the urea and lacking p-phenylene diamine is markedly depressed. Thus the ubiquitous cytochrome oxidase is unaffected by the potent respiratory depressant, n-butyl urea.

TABLE IX

Addition of p-phenylene diamine (PPD) to brain slices with and without n-butyl urea 0.10M. No glucose added
Concentration of PPD expressed in mg. per 3 cc.

	n-Butyl urea Q_{O_2}	None Q_{O_2}
PPD 19 mg.	20.3 23.8	22.0
PPD 25 mg.	25.3 21.7	23.0
None	1.2 0.6	7.2 6.9

(c) As explained in the section devoted to Methylene Blue Reduction, the dye, <u>methylene blue</u>, can act as a hydrogen acceptor receiving hydrogen from the coenzyme-dehydrogenase complex. The reduced dye, leuco form, is readily re-oxidized or dehydrogenated by molecular oxygen and is thus a reversible redox system under aerobic conditions. Since the cell utilizes a terminal respiratory complex (cytochrome-cytochrome oxidase) which is capable of oxidizing most of the metabolic hydrogen produced by the carrier-dehydrogenases, any increase in oxygen consumption elicited by the addition of the dye

(acceptor respiration) would be small. If, however, the terminal respiratory system be poisoned by carbon monoxide, cyanide or any specific inhibitor, acceptor respiration with methylene blue would be relatively large, since competition for the metabolic hydrogen would be minimized (Barron 1929, 1930). It will be noted that the integrity of the carrier-dehydrogenase is essential for any manifestation of acceptor respiration.

The addition of 0.01% of methylene blue to slices of brain respiring in the presence of n-butyl urea had no appreciable effect on the depressed rate of oxygen consumption. On the basis of the foregoing discussion, this result indicates that the site of depressant action of n-butyl urea is located in the carrier-dehydrogenase phase of the respiratory enzymatic chain and not in the terminal cytochrome-cytochrome oxidase domain. The results of the experiments with p-phenylene diamine and methylene blue are highly informative of locus of depressant action. Their modes of operation are dissimilar, yet they yield corroborating evidence, i.e. that the cytochrome-cytochrome oxidase system is not affected by n-butyl urea and indicate the carrier-dehydrogenase complex as a possible recipient of inhibition.

(d) Succinic acid. The important position of the dicarboxylic acid, succinic, in cellular respiration has been outlined in the introduction to this work. From the mass of investigation of this compound, it has been found by the use of various respiratory inhibitors that succinic acid is oxidized to fumaric acid by means of the omnipresent succinic dehydrogenase

in cooperation with cytochrome-cytochrome oxidase. Most investigators deny the necessity of an intermediate coenzyme or "carrier" (Szent-Gyorgyi, 1939; Quastel and Weatley, 1932, 1934; Greville, 1936, 1937; Stare and Bauman, 1938; Jowett and Quastel, 1937; Potter and Elvehjem, 1937). The results of recent studies of the succinoxidase system have suggested the presence of a flavin (Axelrod, Potter, Elvehjem, 1942) and the participation of calcium and a trivalent ion, possibly aluminum (Potter and Schneider, 1942). Despite uncertainty concerning the full complement of the succinoxidase complex, succinic dehydrogenase, cytochrome, and cytochrome oxidase (Atmungsferment, indophenol oxidase) are operative in the oxidation of succinic acid.

In view of the results obtained with p-phenylene diamine and methylene blue, the relative and absolute immunity of the terminal respiratory system (cytochrome-cytochrome oxidase) from the inhibitory action of n-butyl urea has been demonstrated. In order to determine the influence of the urea on the succinic dehydrogenase, succinic acid may be added to tissue previously depressed with n-butyl urea. An unchanged rate of respiration would suggest inhibition of the dehydrogenase, whereas an increase in rate would prove its relative immunity. This plan was executed and the results are presented in Table X.

Tissue slices were placed in the usual Ringer-phosphate media which contained n-butyl urea but no added glucose. After the respiratory rate had been determined over a 30 minute interval, succinic acid (neutralized with sodium

hydroxide to pH 7.3) was added and the rate followed for 60 to 90 minutes.

In the tissues examined (brain, tumor, liver, muscle, and kidney) there occurred a reversal of rate with a marked rise in Qo_2. In absolute increase, tumor is far inferior to the normal tissues, and this fact strongly suggests a deficiency of succinic dehydrogenase in the tumor. The large increase in oxygen uptake following addition of succinate

TABLE X

The addition of succinate 0.04M to tissue slices treated with n-butyl urea 0.1M in absence of added glucose.

	Det'n	n-butyl urea Qo_2	Add. succ. Qo_2
Brain	15	1.1	7.1
Tumor	7	1.3	4.0
Liver	5	4.9	12.1
Muscle	4	2.5	9.8
Kidney	5	4.7	34.0

demonstrates the relative integrity of the complete succinoxidase system in the presence of n-butyl urea.

These results were obtained with a medium to which no glucose had been added. When glucose was included in the original media with n-butyl urea, addition of succinate failed to produce as marked an increase in Qo_2. This discrepancy was observed only in brain and tumor. Subsequent sections of this paper deal largely with an explanation and interpretation of this problem.

INHIBITION OF SUCCINIC ACID OXIDATION

In the foregoing section which dealt with the reversal of respiratory inhibition, it was found that while the oxidation of glucose was depressed by n-butyl urea, that of succinic acid was not. Adding glucose to a tissue respiring at a low level in the presence of the urea, elicited no change in rate, since the enzyme system involved in the oxidation of

TABLE XI

The effect of 0.20% glucose on the oxidation of 0.04M succinic acid in the presence of 0.1M n-butyl urea.

Tissue	Col.I No. Det'n.	Col.I n-butyl urea succinate glucose Q_{O_2}	Col.II No. Det'n.	Col.II n-butyl urea succinate --- Q_{O_2}	I/II x 100 %
Brain	22	4.9	15	7.1	68
Tumor	8	2.2	7	4.0	55
Liver	6	11.2	5	12.1	93
Muscle	5	10.2	4	9.8	104
Kidney	4	34.7	5	34.0	102

glucose has been shown to be inhibited. The addition of succinic acid to a similarly treated tissue caused a marked increase in respiratory rate, demonstrating the immunity of the succinoxidase. When, however, succinic acid and glucose were added to certain tissues in the presence of n-butyl urea, a much smaller increase in Q_{O_2} occurred.

Comparison of Tissues

Table XI presents the result of a series of Q_{O_2} determinations of various tissues which had previously respired in a

Ringer-phosphate medium containing n-butyl urea, 0.1M, with or without 0.20% glucose. After 30 minutes of this preliminary period, 0.04M succinic acid was added and the respiratory rate was measured over a 60 minute interval. It is the Qo_2 following the advent of succinic acid which is recorded.

Succinic acid oxidation was not appreciably affected by the presence of glucose in muscle (diaphragm), kidney, or liver; however, in brain and tumor, the addition of glucose

TABLE XII

Effect of glucose on succinic oxidation in the absence of n-butyl urea. Glucose 0.2%, succinate 0.04M. Brain slices.

		glucose	glucose	none	none
Control period	Qo_2	9.7	9.7	6.5	5.5
Succinate addition	Qo_2	14.2	14.1	8.7	8.1
Increase	Qo_2	4.5	4.4	2.2	2.6
Increase	%	46	45	34	47

caused a significant decrease in Qo_2. In other experiments with brain tissue in which n-butyl urea had not been employed, the presence of glucose had no depressing influence on the utilization of succinic acid. This data has been analyzed in Table XII. In order to obtain this anomalous behavior, the urea must be present even though it exerts no demonstably direct effect on the succinoxidase system.

A search of the voluminous literature of Biological Oxidations revealed no mechanism whereby glucose or its oxidizing enzymes might interfere with the oxidation of succinic

acid in cellular respiration, it was decided to attempt an elucidation of this reaction common to brain and tumor using n-butyl urea as a necessary reagent.

Glucose Concentration

The inhibitory influence of glucose or its intermediate metabolic products on the oxidation of succinic acid has been demonstrated qualitatively. In order to study the reaction quantitatively, glucose was added to the respiratory medium in a series of graded concentrations from 0.01 to 0.30%. n-Butyl urea, 0.1M, was included in the medium and 0.04M succinic acid added as previously described.

Typical results of such a procedure with brain, tumor, and liver are embodied in Figure 4. In brain and tumor, the tissues showing the oxidative inhibition of succinate, an inverse proportion exists between the glucose concentration and the Q_{O_2} (succinate oxidation). It has been found that there was a limiting concentration of glucose beyond which no further diminution of Q_{O_2} was noted. This value ranges from 0.25 to 0.35%; the precise concentration varies from tissue to tissue depending on the metabolic state of the cells and other factors. The results obtained with liver are at variance with those with brain and tumor. The effect of glucose on liver Q_{O_2} is one of enhancement rather than inhibition.

Non-specificity of the Reagent

In order to produce the inhibition of succinic acid oxidation, in the presence of glucose, it has been found that n-butyl urea must also be added although it apparently has

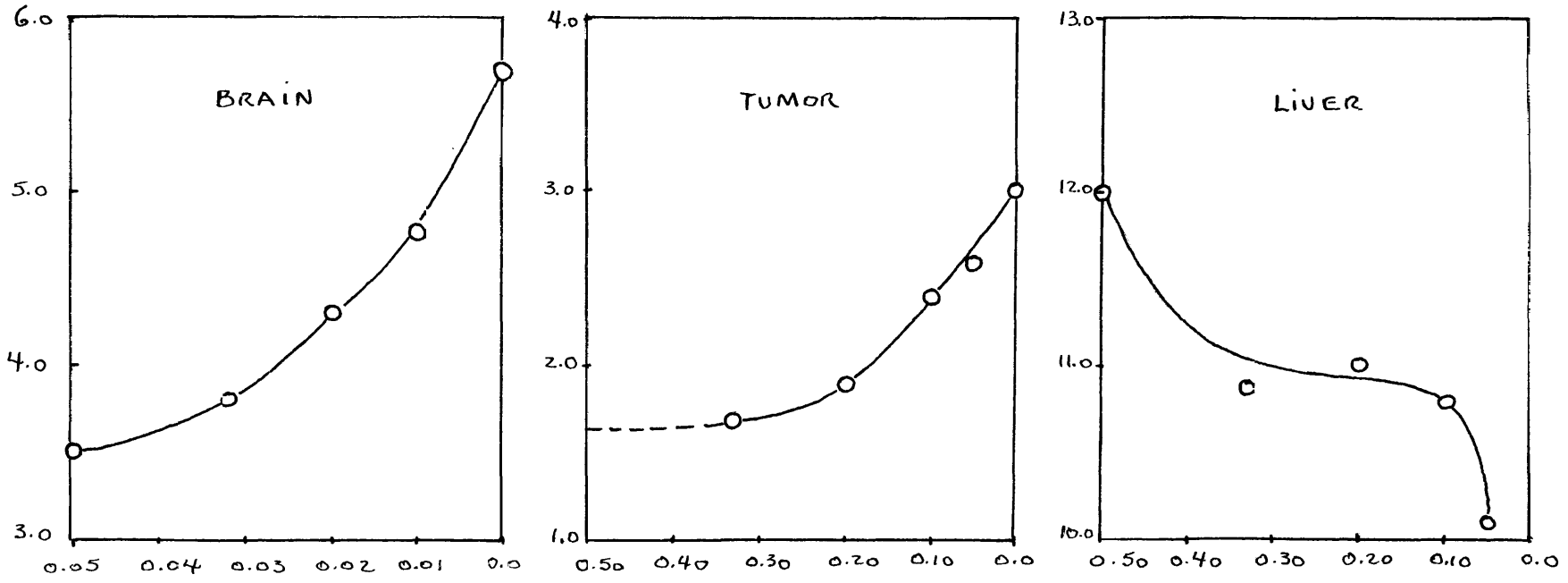
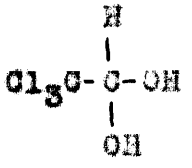


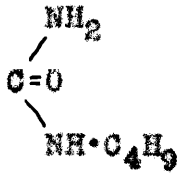
Figure 4

The influence of varying concentrations of glucose on the oxidation of succinic acid, 0.04M with n-butyl urea present. Abscissae, glucose in percentage. Ordinates, Qo_2.

no direct effect on the succinoxidase. To determine whether n-butyl was specifically required was accomplished by substituting chloral hydrate, a narcotic agent of an entirely different chemical constitution, for the urea. It was found



chloral hydrate



n-butyl urea

that chloral hydrate 0.30% caused a marked depression in the Qo_2 of brain and in this pharmacological aspect was similar to the urea.

In a series of 3 experiments in which the media contained chloral hydrate and succinic acid, the presence of glucose occasioned an average Qo_2 which was but 75% of that of the glucose free media. Thus the results with chloral hydrate closely resemble those with n-butyl urea. Chloral hydrate was employed because of its chemical dissimilarity to n-butyl urea. The substitution of a barbiturate would not afford the same degree of cruciality owing to the chemical relationship between it and the urea.

Comparison of Carbohydrates

In explaining the inhibition of the succinoxidase system it was of interest to know whether the action of glucose was specific. Accordingly, a number of carbohydrates and derivatives were substituted for glucose and the oxidation of succinic acid in brain determined as before. When the influence

of a carbohydrate was to be ascertained, a control with no added carbohydrate was run simultaneously. Results were expressed as the percentile relation of the former to the latter, i.e.,

$$\frac{Q_{O_2} \text{ of carbohydrate containing sample}}{Q_{O_2} \text{ of control sample}} \times 100$$

The influence of 16 common carbohydrates and derivatives on the oxidation of succinic acid in the presence of n-butyl urea was determined on brain slices. The experimental measurements were made in precisely the same manner as those described with glucose. Table XIII summarizes the results obtained and includes determinations with glucose to afford a basis of comparison.

Of the 16 compounds examined the only marked inhibition of Q_{O_2} (succinate oxidation) was induced by glucose. The respiratory rate in the presence of this monosaccharide was 68% of that in its absence. Mannose produced a slight inhibition of succinic acid oxidation or 88% of the control value.

Of the substances examined, fructose, mannose, sorbitol, mannitol, lactate and pyruvate are metabolized to some degree by isolated cells and, therefore, share this property with glucose. An even closer relationship exists in the case of glucose, fructose and mannose, which is manifested in the well known equilibrium mixture of these monosaccharides through the intermediation of a common enol form (Armstrong and Armstrong, 1934). A number of investigators (Oppenheimer and Stern, 1939) have suggested that the primary product of

TABLE XIII

The influence of carbohydrates and derivatives on the oxidation of succinic acid 0.04M, in brain, in the presence of n-butyl urea 0.1M. Results expressed as percent of controls having no added carbohydrates.

Substance	Conc.	No. Det'n.	% of control
Glucose	0.20%	20	68
Fructose	0.20%	4	111
Mannose	0.20%	6	88
Sorbose	0.20%	1	119
Galactose	0.20%	4	108
Trehalose	0.20%	2	108
Sorbitol	0.20%	2	100
Mannitol	0.20%	1	100
Dulcitol	0.20%	3	108
Xylose	0.20%	2	99
Arabinose	0.20%	1	112
Rhamnose	0.20%	1	111
Lactate	0.0125M	6	126
Pyruvate	0.125M	3	110
Gluconate	0.20%	2	102
Hexose di PO_4	0.20%	2	105

glucose breakdown is the enol form which is stabilized by enzymatic phosphorylation in the number 6 carbon atom position yielding a hexose phosphate. In such a scheme, the cell should not discriminate among glucose, fructose, and mannose in their qualitative oxidizability. Possibly a difference in conversion velocity may explain the quantitative difference observed by many in both in vivo and vitro oxidation of these hexoses and also in the inhibition of succinate oxidation under discussion.

The failure of lactic and pyruvic acids, neutralized with sodium hydroxide, to inhibit succinate oxidation was surprising in view of their positions as theoretical intermediary products of glucose degradation. Quastel and Wheatley, 1932, investigated the influence of sodium lactate on succinic acid oxidation and noted a slight inhibition which they termed the "sparing action of lactate." Their attempts to uncover the mechanism of the reaction were unsuccessful. This "sparing action" could be demonstrated in the absence of a depressant substance but not in the presence of such an agent.

Order of Addition

In all previous measurements of succinoxidase inhibition by glucose, the succinic acid was added to the tissue respiring in a Ringer-phosphate medium containing n-butyl urea and glucose. The succinate was always added 30 minutes after the beginning of the experiment. In this section, various orders or addition have been studied together with the element of

time. In each evaluation of succinate inhibition one measurement was made in the presence of glucose and a parallel one in its absence.

1. Time relationship. Table XIV presents the results of a series of determinations of succinic acid oxidation in brain and tumor slices. The order of addition of constituents was identical with that of the previous plan, as mentioned above.

TABLE XIV

Effect of the elapsed time between the additions of glucose and succinate on the oxidation of the latter. n-Butyl urea present.

		Time elapsed min.	Qo_2	% of control
Brain	glucose	0	4.9	70
	none	0	7.0	--
	glucose	15	4.4	72
	none	15	6.1	--
	glucose	45	5.0	66
	none	45	7.6	--
Tumor	glucose	0	3.3	56
	none	0	5.9	--
	glucose	45	2.7	50
	none	45	5.4	--

The variable factor was the time which elapsed at the start of the experiment, and the subsequent addition of succinate. Measurements of Qo_2 began with the advent of succinate in the media. The results with each time interval were similar and closely resembled those obtained in previous work with the 30 minute hiatus. Thus, the time factor in this order of addition is relatively unimportant.

2. Glucose following succinate. As another variation in the

pattern, glucose was added to the medium 30 minutes after the addition of succinate and n-butyl urea. A typical series is arranged in Table XV. Glucose, even though added after the succinic acid, exerts an inhibitory effect upon the latter. Some depression of the succinoxidase system is present although much less than that when glucose was added before the succinate.

TABLE XV

Succinic acid oxidation as influenced by the addition of glucose subsequent to that of the substrate and n-butyl urea. Brain slices.

n-Butyl urea	M.	0.1	0.1
Succinate	M.	0.04	0.04
	Q_{O_2}	6.5	6.2
Add. Glucose	%	0.20	0.0
	Q_{O_2}	4.9	5.5
Percent of original		75	89

3. n-Butyl urea following glucose and succinate. Tissue slices were placed in Ringer-phosphate media supplemented with succinic acid and glucose. After 30 minutes, n-butyl urea was tipped in from the side bulbs and Q_{O_2} measurements initiated. Table XVI illustrates such a procedure. Here, as in the preceding condition, glucose produces some inhibition of succinic acid oxidation but is quantitatively inferior to that effected when n-butyl urea was introduced before the substrate, succinic acid.

It has therefore been established that the maximum inhibition of succinoxidase is produced by the addition of

succinic acid to media already containing glucose and n-butyl urea. Whether the actual cause of inhibition be (1) direct adsorption complex of glucose or its enzymes on the succinoxidase or (2) toxic product of glucose degradation, either normal or abnormal, the inhibition reaction is favored by the advent of glucose simultaneous or prior to that of succinate.

TABLE XVI

Succinic acid oxidation as influenced by the addition of n-butyl urea subsequent to that of the substrate glucose. Brain slices.

Succinate M.	0.04	0.04
Glucose %	0.20	0.00
Qo_2	14.1	12.3
Add.n-butyl urea	0.1	0.1
Qo_2	6.0	7.2
Percent of original	43	59

Brain Brei

When fresh brain was ground in a mortar and suspended in 5 volumes of distilled water, the resulting suspension or brei contained no intact cells on microscopic examination. The combination of mechanical agitation and hypotonic cytolysis reduced the tissue to a colloidal suspension and cellular debris. Such a brei exhibited a low, residual respiration which failed to increase appreciably when glucose was added. A typical figure for the residual respiration is 7.4 cu. mm. of oxygen per hour and with glucose, 7.5 cu. mm. of oxygen. Approximately the same results were obtained with a liver brei.

The reason for the inability to utilize glucose may possibly be the dilution factor or the spatial separation of enzyme complex from substrate. Since many active cellular enzymes are readily extracted and partially purified, usually after rather drastic treatment, it does not seem likely that the procedure followed above would suffice to inactivate an individual enzyme, per se.

Inasmuch as brain brei could utilize succinic acid and not glucose, it was decided to determine the influence of

TABLE XVII

The influence of glucose on succinic acid oxidation in brain brei. Oxygen consumption expressed as cu. mm. per hour. n-Butyl urea present.

Brei	Glucose	None
1.0 cc.	131.0	129.0
0.5 cc.	43.7	41.2
0.5 cc.	42.3	40.3

the metabolically inert glucose on succinate oxidation in the presence of n-butyl urea. Table XVII summarized the results.

In contradistinction to results obtained with brain slices, no inhibition of succinate oxidation by glucose was present in brei. Undoubtedly this difference in reaction may be entirely accounted for by the absence of glucose metabolism in this cell state. Despite the abnormality of the preparation, an obvious inference may be drawn. In order for glucose to effect inhibition of succinic acid oxidation, the enzyme complex responsible for the metabolism of glucose must be spatially intact, although it is partially inhibited

by n-butyl urea.

These results are suggestive of the relative complexity of the glucose and succinic acid oxidizing systems. It is reasonable to assume the immunity of individual enzymes to the processes of brei formation and attribute enzymatic failure to molecular or colloidal disorientation. Since both metabolites require the participation of at least two enzymes or "carriers," an interruption in their spatial relationship, e.g. dilution, would necessarily render them less efficient. It therefore follows that the system displaying more extensive inhibition in the brei preparation is the one of greater complexity. Thus it would appear that the oxidizing complex of glucose is constituted of more individual participants than that of succinic acid, in view of the almost complete failure of glucose utilization as contrasted to the rapid oxidation of succinate.

Methylene Blue Reduction Time of Succinate

An earlier section contains a description of the mechanism of methylene blue reduction and its assay in the Thunberg tube. Since the dye reduction involves a relatively simple enzymic pattern, results are often informative.

Succinic acid 0.04M, n-butyl urea 0.1M, and glucose 0.2% were placed in the tubes together with brain and media; and the effect of glucose on the reduction time of methylene blue by succinate was measured. The results, given in Table XVIII reveal the absence of succinate inhibition by glucose. In this technique which is conducted under strictly anaerobic

TABLE XVIII

Methylene blue reduction time of succinic acid as influenced by glucose. Brain suspension.

Constituents			Reduction time min.	min.	aver.
Glucose	Butyl	----	150	-	150
---	---	Succ.	17	15	16
Glucose	---	Succ.	12	10	11
---	Butyl	Succ.	30	29	28
---	Butyl	Succ.	30	23	
Glucose	Butyl	Succ.	29	23	28
Glucose	Butyl	Succ.	26	32	

TABLE XIX

The effect of glucose on the oxidation of p-phenylene diamine (PPD) in brain. n-butyl urea present.

PPD in media	glucose	aver.	none	aver.
	Q_{O_2}	Q_{O_2}	Q_{O_2}	Q_{O_2}
19 mg.	19.8	20.2	20.3	22.1
	20.6		23.8	
25 mg.	31.6	28.2	21.7	23.5
	24.7		25.3	

conditions, only a portion of the enzymic chain is operative. In view of the results it would appear that the presence of the aerobic enzymes in active form is a requisite for succinate inhibition by glucose.

Glucose and p-Phenylene diamine

p-Phenylene diamine shares with succinic acid the immunity to oxidative inhibition by n-butyl urea. While the former is not in itself indigenous to living tissue its utilization by the cell entails a physiological mechanism. Since it has been demonstrated that glucose in the presence of n-butyl urea exerts an inhibitory influence on succinic acid oxidation, substitution of p-phenylene diamine for succinate was undertaken. The results are presented in Table XIX.

Qo_2 values obtained with p-phenylene diamine must be confined to readings of oxygen consumption over a relatively small interval because of the extreme instability of the molecule.* Thus these factors accentuate individual variations. However, no evidence for appreciable inhibition of p-phenylene diamine by glucose is present.

The depressant action of glucose, as shown by succinate inhibition, is inoperative against p-phenylene diamine, under the same conditions. Since the oxidation of the latter is an index of function of the terminal respiratory chain utilized by succinic acid, its immunity to depression suggests that glucose inhibits succinic acid metabolism in the primary phase,

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* See earlier section devoted to this compound.

possibly at the dehydrogenase.

Hydrogen-ion Concentration

In the presence of a phosphate buffer, the pH of respiratory media changes very slightly when tumor and brain oxidize either glucose or succinate. However, determinations of hydrogen-ion concentration of media showing the inhibition of succinic acid oxidation by glucose in the presence of n-butyl urea revealed increased acidity. Table IX presents the results of this procedure with brain and tumor. All media contained n-butyl urea, 0.1M, and succinic acid, 0.04M; but glucose, 0.2%, was present in only half the cases. Qo_2 measurements were made in the customary manner and pH estimated by use of the Beckman pH meter.

It will be noted in Table IX in the portion of results derived from brain and tumor in the presence of calcium that the addition of glucose caused directly or indirectly (1) inhibition of succinate oxidation, and (2) a marked pH decrease in media. The glucose free tissues had a pH value close to that of the original media, 7.3, whereas those with added glucose were often as low as pH 6.8.

In order to ascertain whether the increased acidity of the tissues and media was a contributing cause of succinate inhibition or merely an effect, media were prepared with a series of pH values from 7.3 to 6.5. These media were of the same composition as those used throughout this with the exception of the pH of the phosphate buffer. n-Butyl urea was added to this series with succinic acid but glucose was omitted.

TABLE XX

Influence of Calcium, 0.003M, and pH of media on succinate inhibition by glucose. n-butyl urea present.

		No glucose		Glucose	
		Qo_2	pH	Qo_2	pH
Brain	Calcium	7.7	7.25	5.1	6.86
		8.1	7.23	4.3	6.76
		10.8	7.32	5.7	6.98
		8.5	7.22	5.6	6.78
		10.6	7.25	6.0	6.90
		8.5	7.25	6.3	6.97
	No Calcium	10.0	7.21	9.6	6.88
		10.3	7.35	9.1	6.96
Tumor	Calcium	4.1	7.18	1.9	6.49
		3.5	7.21	1.2	6.50
	No Calcium	2.8	7.12	2.5	6.84
		1.8	7.20	2.1	6.70

It was found that at pH 6.5 to 6.9 succinate oxidation was inhibited in the absence of glucose. Thus lowered pH, as a result of the presence of glucose, may be looked upon as an accessory in the succinic acid inhibition reaction.

Influence of Calcium

As described in the preceding section, Ringer-phosphate media of various pH values were prepared for the purpose of simulating glucose inhibition of succinic acid. In addition to the usual Ringer-phosphate solution, saline or 0.15M sodium chloride and a borate buffer mixture were also employed as media. This latter solution has been extensively employed by Feinstein and Stare (1940) and was compounded in accordance with their directions. It contained boric acid, sodium and potassium chlorides and was adjusted to pH 7.3 with sodium hydroxide.

When brain was added to either of these media together with n-butyl urea and succinate, it was discovered that glucose had no appreciable effect on the oxidation of the succinate although pH was markedly lowered in the presence of glucose. It was, therefore, assumed that acidity was not the entire cause for succinate inhibition by glucose.

A comparison of the composition of the three media used, i.e. Ringer-phosphate, saline and borate, revealed that calcium which was contained in Ringer-phosphate was lacking in the others. Accordingly a modified Ringer-phosphate media was prepared to which no calcium was added. Where possible, calcium free salts were used as the necessary ingredients.

The Qo_2 of brain and tumor slices was measured in the presence of succinic acid, n-butyl urea with and without glucose. Table XX summarized the results when carried out in calcium free Ringer-phosphate and the customary Ringer- phosphate containing calcium.

In calcium free media, with both brain and tumor, the addition of glucose produced insignificant changes in Qo_2 of succinic acid, although the pH fell sharply. When calcium was replaced, the usual inhibition of succinic acid oxidation by glucose was manifested. It is, therefore, apparent that the inhibition reaction requires calcium acting in the presence of subnormal pH. Relatively high hydrogen-ion concentration has, per se, little effect on the functioning of the succinoxidase system.

To determine the influence of calcium on succinic oxidation in the absence of n-butyl urea the series assembled in Table XXI was prepared. It will be noted that the addition of calcium produced no appreciable change in the Qo_2 of succinic acid, either in the presence or absence of glucose. The pH of all media was approximately 7.3 throughout the series.

The results appearing in Table XXII were assembled for the purpose of demonstrating the non-specificity of calcium in the succinate oxidation inhibition reaction. In this procedure the salt to be assayed was substituted for calcium chloride and all other components of the system maintained as usual. The divalent cations, strontium, barium, and magnesium, produced succinic acid inhibition similar to that with calcium. Attempts to substitute the trivalent aluminum ion for calcium were not conclusive, owing to the insolubility of the former

in alkaline solution. The negative sulfate ions which are so effective in protein precipitation, particularly as the ammonium salt, were without effect.

Various Media

Mention has been made of borate and saline solutions and their adoption as respiratory media in succinate oxidation.

TABLE XXI

The effect of Calcium, 0.003M, on succinic acid oxidation in the absence of n-butyl urea.

	No Glucose Qo_2	Glucose Qo_2
Calcium	9.2	17.7
	8.9	17.0
	9.4	
	10.6	
	10.4	
average	9.7	17.4
No Calcium	8.8	18.8
	9.7	
	10.6	
average	9.7	18.8

Another type of media was prepared by combining sufficient sodium chloride with M/15 phosphate buffer, pH 7.3, to render the resultant mixture isotonic to serum. The influence of glucose and calcium on succinic acid oxidation of brain in these media was compared to that in the customary Ringer-phosphate, Table XXIII. Since previous work had been carried out in the latter environment, it was decided to study the reaction in media of different and less physiological composition.

TABLE XXII

The effect of divalent and trivalent ions, 0.003M, on succinate inhibition by glucose. n-butyl urea present.

		No glucose		Glucose	
		Qo_2	pH	Qo_2	pH
Brain	Control	10.0	7.21	9.6	6.88
	$SrCl_2$	8.3	7.13	5.3	6.85
		6.7	-	5.3	-
	$BaCl_2$	9.2	-	6.3	-
		9.0	-	6.6	-
	$MgCl_2$	10.5	7.15	7.3	6.75
		12.6	7.20	9.1	6.80
		10.1	7.12	6.6	6.91
	K_2SO_4	9.5	-	8.8	-
	$(NH_4)_2SO_4$	10.9	-	10.0	-
	$AlCl_3$	9.2	-	10.8	-
		10.2	-	8.7	-
Tumor	Control	2.8	7.12	2.5	6.84
	$BaCl_2$	3.5	7.17	1.8	6.81
	$MgCl_2$	3.7	-	2.0	-

TABLE XXIII

The influence of calcium, 0.003M, on the inhibition of succinic acid by glucose in various media. n-butyl urea. Brain.

Media	No Calcium		Calcium	
	Glucose Qo_2	None Qo_2	Glucose Qo_2	None Qo_2
Saline	7.2	6.7	4.3	6.8
	10.6	9.7	4.6	7.8
	-	-	4.0	6.1
	-	-	5.6	11.6
	-	-	4.6	8.6
average	8.9	8.2	4.6	8.6
Ringer-Phosphate*	9.6	10.0	5.7	8.5
	9.1	10.5	5.8	8.7
	-	-	4.7	7.7
	-	-	4.7	8.4
	-	-	4.6	6.4
average	9.4	10.3	5.1	7.9
Phosphate	12.5	10.7	9.0	12.0
	11.1	8.5	10.7	10.8
	9.6	8.8	9.1	8.0
	9.8	11.0	11.0	10.3
	10.0	10.6	10.3	10.1
	11.9	10.3	-	-
	8.5	9.9	-	-
average	10.5	9.5	10.1	10.6
Borate	9.4	7.8	7.0	13.4
	7.8	7.9	6.0	11.1
	9.2	7.9	-	-
	8.5	9.6	-	-
average	8.7	8.3	6.5	12.3

* Calcium in Ringer-phosphate was 0.0005M.

The results with saline, borate and Ringer-phosphate were essentially the same. Inhibition of succinate oxidation occurred only in the presence of glucose and calcium in these media. Since both borate and saline contained no phosphate, the participation of phosphate ions in glucose degradation would appear unnecessary. This point is, however, subject to debate since the tissue slices may cling tenaciously to traces of the element despite attempts to remove it. The essential fact is that phosphate, if present at all, existed at a definitely subnormal level.

The combination of glucose and calcium failed to effect inhibition of succinic acid in the strongly buffered phosphate solution. pH values obtained from this medium revealed that the production of acid from glucose caused no significant drop in pH. This fact alone explained the lack of inhibition in the strong phosphate medium.

Aerobic Glycolysis

Attention was directed to the acid derived from glucose in the presence of n-butyl urea and succinate. The media yielded a negative nitroprusside test, but a positive Kelling's test for lactic acid was obtained. Quantitiative determinations were instituted using the method of Freidemann, Cotonio, and Shaffer (1927), and the results with brain and tumor were tabulated in Table XXIV. The values are expressed as mg. lactic acid per gm. tissue and represent the production during a 90 minute period (aerobic glycolysis).

The primary purpose of these determinations was to measure

the increased acidity which arises from glucose in the presence of n-butyl urea and assists in succinate oxidation inhibition. Attention is called to parts (3) and (4) of Table XXIV in both brain and tumor. The presence of glucose (4) with n-butyl urea and succinate was responsible for a lactic acid production of 52.0 and 72.0 mg. per gm. per 90 minutes in brain and tumor respectively. In the absence of glucose (3) the acid accumulated at the rate of 12.0 and 14.0 mg. This rather

TABLE XXIV

Lactic acid production and resultant pH of respiratory media.

		Constituents			Lactic acid per gm. per 90 min. mg.	pH
Brain	(1)	---	---	Succ	2.0	7.32
	(2)	Glucose	---	Succ	12.0	7.30
	(3)	---	Butyl	Succ	12.0	7.28
	(4)	Glucose	Butyl	Succ	52.0	6.70
Tumor	(1)	---	---	Succ	9.0	--
	(2)	Glucose	---	Succ	27.0	--
	(3)	---	Butyl	Succ	14.0	7.20
	(4)	Glucose	Butyl	Succ	72.0	6.53

unexpected acceleration of aerobic glycolysis by n-butyl urea accounted for the lowered pH of the media, as will be shown.

Chemically pure lactic acid was added to the usual respiratory media in varying amounts and the resultant pH values plotted against lactic acid concentration on graph paper. It was found that the results of Table XXIV fitted this titration curve very closely and thus explained the pH in terms of lactic acid.

DISCUSSION

Action of Urea Derivatives

In general the influence of derivatives of urea on *in vitro* tissue respiration was one of depression which increased with molecular weight. Certain ureas, usually of low molecular weight, produced a preliminary stimulation of Qo_2, but this effect was soon replaced by depression.

The various tissues examined displayed variations in reaction to these agents. Those possessing a high Qo_2 were often inhibited more than those respiring at a lower rate. The order of tissues with respect to resistance to depression to the typical n-butyl urea was as follows: muscle, liver, tumor and brain, kidney.

With regard to possible chemotherapeutic application in neoplastic pathology, one compound examined which evidenced promise was n-propyl urea. In *in vitro* studies of Qo_2, those normal tissues were but slightly depressed, whereas that of tumor was lowered somewhat more. Owing possibly to the individual variations inherent in such measurements, statistical analysis by means of Probable Error gave a "slight significance" to the difference in degree of respiratory inhibition in normal and tumor tissues. Despite the occasionally unbridgeable gulf separating results obtained from *in vivo* and *vitro* studies, the influence of n-propyl urea on tumor bearing animals should be tested.

Mode of Action

In determing the mechanism and locus of action of the ureas, recourse has been made to special systems and reagents. Mere observation of Q_{O_2} inhibition by a urea discloses little information concerning the mechanism involved. In the anaerobic methylene blue reduction technique, only the initial respiratory enzyme chain is operative. Thus the retardation by ureas of dehydrogenation time in the various tissues indicates inhibition of the dehydrogenase-coenzyme complex.

That the terminal respiratory system of cytochrome-cytochrome oxidase is immune to urea depression was evidenced by the lack of influence of n-butyl urea on the oxidation of p-phenylene diamine. Since the utilization of this latter compound is entirely dependent on the cytochrome complex, any damage to this system would naturally be manifested in the oxidation of the diamine. Another line of evidence for freedom from depression was afforded by the unimpaired oxidation of succinic acid in the presence of ureas. This metabolite requires only a specific dehydrogenase and the cytochrome complex for oxidation.

From this and other evidence presented in the text, the locus of the depressant action of ureas is assigned to the initial respiratory enzyme complex (the dehydrogenase-coenzyme) which is responsible for the activation and removal of hydrogen from the metabolite.

The events occurring in Q_{O_2} depression under the influence of n-butyl urea may be pictured in the following manner:

(1) The aerobic glycolytic mechanism which is uninhibited

converts glucose to lactic acid.

(2) Since both glucose and lactic acid dehydrogenases are depressed, neither metabolite is dehydrogenated and oxidation ceases.

(3) Glycolysis continues with the accumulation of lactic acid and the concomitant disappearance of glucose.

Succinate Inhibition

It has been shown that the oxidation of succinic acid is inhibited by glucose in the presence of n-butyl urea. Reference was earlier made to the oxidizing system of succinic acid which is composed of a dehydrogenase coupled with the cytochrome-cytochrome oxidase. Since the latter component is immune to the inhibition involving succinate as demonstrated by p-phenylene diamine oxidation, the dehydrogenase is implicated as the site of depression.

In the preceding discussion, the mechanism for obtaining the acid condition of the media requisite for succinate inhibition was defined. The means by which calcium, action at pH 6.5 to 6.8, inhibits the succinic dehydrogenase, is however, not entirely understood. The possibility of flocculation of the colloidal dehydrogenase by divalent cations was examined. It was reasoned that since the stability of emulsoids is diminished as the iso-electric point is approached, particularly in the presence of certain ions, that such a set of conditions might be effective in this case. As given by Ogston and Green (1935) the iso-electric point of succinic dehydrogenase is approximately pH 4.8. Since the pH of the inhibition media

was usually 0.7 of a unit nearer pH 4.6 than that of the normal media, colloidal instability may be proportionately increased. Existing at a pH above its iso-electric point, the protein dehydrogenase would carry a negative charge. The fact that divalent cations and not anions effected succinic acid inhibition would tend to support this view. Obviously further investigation is required to explain the observations.

In view of the similarity in action between the urea derivatives and the narcotics, it would be anticipated that during narcosis brain cytoplasm would suffer a fall in pH by virtue of lactic acid accumulation. The relationship between the blood calcium and that in brain has not been completely described; but the presence of approximately 36 mg. calcium per 100 gm. fresh rat brain (Hess, Gross, Weinstock, and Berliner 1932), even partially in the ionic state, would suffice to foster succinate inhibition. Various psychic phenomena (amnesia, mental sluggishness, sleep, etc.) and their possible relationships to the attendant calcium and hydrogen-ion increases is reviewed by Page (1939,,pp. 217-245). Conceivably such correlations may be partially explained on the basis of inhibition of the vital succinic dehydrogenase.

Mention should be made of the use of Coenzyme I in the succinate inhibition reaction. Diphosphopyridine nucleotide was extracted from brewers' yeast by the method of Williamson and Green (1940). When traces were added to glucose, n-butyl urea, and succinic acid, the usual oxidative inhibition of the last substance was not observed. This procedure was not studied in detail and, therefore, requires further investigation.

The results, however, suggest participation of the coenzyme in the inhibition of succinoxidase.

CONCLUSIONS

1. The derivatives of urea examined depress the oxygen consumption and anaerobic dehydrogenation of normal and tumor (Walker rat Sarcoma #319) tissues. In general, this depression is proportional to the molecular weight of the compound.
2. The oxidation of 20 common carbohydrates and derivatives is inhibited.
3. Succinic acid (succinic dehydrogenase) and p-phenylene diamine (cytochrome complex) are not influenced.
4. Anaerobic glycolysis is slightly stimulated in normal tissues by the ureas but somewhat depressed in tumor, whereas aerobic glycolysis in brain and tumor is markedly accelerated by n-butyl urea.
5. By the use of special preparations and reagents, the locus of respiratory depression was restricted to the dehydrogenase-coenzyme complex.
6. The oxidation of succinic acid is inhibited by glucose in the presence of n-butyl urea or certain other depressant agents. This result is effected by the increased lactic acid production and the available calcium ions. An interpretation of this reaction is presented and its possible relation to narcosis discussed.

LITERATURE CITED

Armstrong, E.F. and K.F. Armstrong. "The Carbohydrates," Longmans, Green and Co., New York, 1934.

Ashford, C.A. J. Biol. Chem. 27, 903 (1933).

Axelrod, A.E., V.R. Potter and C.A. Elvehjem. J. Biol. Chem. 142, 85 (1942).

Baker, L., J.F. Fazikas and H.E. Himwich. J. Biol. Chem. 125, 545 (1938).

Bandrowski, E. Ber. 27, 480 (1894).

Barron, E.S.G. J. Biol. Chem. 81, 445 (1929).

Barron, E.S.G. J. Exp. Med. 52, 447 (1930).

Dickens, F. and G.D. Greville. Biochem. J. 27, 832 (1933).

Dixon, M. in "Perspectives in Biochemistry," Cambridge University Press, Cambridge, England, 1938.

Dodds, E.C. and G.D. Greville. Nature 132, 936 (1933).

Ehrenfest, E. and E. Ronzoni. Proc. Soc. Exp. Biol. and Med. 31, 318 (1933).

Elliott, K.A.C. Physiol. Rev. 21, 267 (1941).

Elvehjem, C.A. and P.S. Wilson. "Respiratory Enzymes," Burgess Publishing Co., Minneapolis, Minn., 1939.

Feinstein, R.N. and F.J. Stare. J. Biol. Chem. 135, 393 (1940).

Freidemann, T.E., M. Cotonio and P.A. Shaffer. J. Biol. Chem. 73, 335 (1927).

Greville, G.D. Biochem. J. 30, 877 (1936).

Greville, G.D. Biochem. J. 31, 2274 (1937).

Hess, A.F., J. Gross, M. Weinstock and F.S. Berliner. J. Biol. Chem. 98, 625 (1932).

Himwich, H.E. and J.F. Fazikas. Am. J. Physiol. 113, 63 (1935).

Jowett, M. and J.H. Quastel. Biochem. J. 31, 565 (1937).

Keilin, D. Proc. Roy. Soc. B. 104, 206 (1929).

Ogston, F.J. and D.E. Green. Biochem. J. 29, 1983 (1935).

Oppenheimer, C. and K.G. Stern. "Biological Oxidations." p. 113, Nordemann Publishing Co., New York, 1939.

Page, I.H. "Chemistry of the Brain," Charles C. Thomas, Baltimore, Md., 1937.

Potter, V.R. and C.A. Elvehjem. J. Biol. Chem. 117, 341 (1937).

Potter, V.R. and W.C. Schneider. J. Biol. Chem. 142, 543 (1942).

Quastel, J.H. and M.D. Whetham. Biochem. J. 18, 519 (1924).

Quastel, J.H. and A.H.M. Wheatley. Biochem. J. 26, 725 (1932).

Quastel, J.H. and A.H.M. Wheatley. Biochem. J. 28, 1521 (1934).

Sherif, M.A.F. and B.C. Holmes. Biochem. J. 24, 400 (1930).

Stare, F.J. and C.A. Bauman. Proc. Roy. Soc. B. 121, 338 (1936).

von Szent-Gyorgyi, A. "On Oxidations, Fermentation, Vitamins, Health and Disease," Williams and Wilkins Co., Baltimore, Md., 1939.

Thunberg, T. Skand. Archiv. Physiol. 20, 1 (1920), Quoted by Page (1937).

Warburg, O. "Uber den Stoffwechsel der Tumoren," Julius Springer, Berlin, 1926.

Whitmore, F.C. "Organic Chemistry," p. 805, D. Van Nostrand Co., New York, 1937.

Williamson, S. and D.E. Green. J. Biol. Chem. 135, 545 (1940).

BIOGRAPHY

The author was born in Bradley Beach, New Jersey in 1915. Secondary education was received at the Grover Cleveland High School in Caldwell, New Jersey. He obtained the Batchelor of Arts degree in 1937 and the Master of Arts one year later from Wesleyan University, Middletown, Connecticut. The period from 1939 to 1940 was spent as research assistant in the Department of Biology of his Alma Mater.

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