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THE INHIBITORY ACTION OF SOME ANTIMALARIAL DRUGS AND RELATED COMPOUNDS ON THE HEXOKINASE OF YEAST AND OF *PLASMODIUM BERGHEI*

BY

DOROTHY M. FRASER* AND W. O. KERMACK

From the Department of Biological Chemistry, University of Aberdeen

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Of various antimalarial compounds tested, only proguanil failed to inhibit yeast hexokinase. The metabolite of proguanil, 10,580, was an effective inhibitor. Some compounds tested which were without antimalarial activity were potent inhibitors of yeast hexokinase. The degree of inhibition increased as the time during which the enzyme had been in contact with the drug increased, and the inhibitory action of mepacrine was reduced when the concentration of ATP was raised. The inhibition of yeast hexokinase by 10,732 was independent of the concentration of ATP.

The hexokinase of haemolysates of the reticulocytes of mouse or rat blood was not appreciably higher than that of similar haemolysates of normal erythrocytes. Preparations of mouse or rat erythrocytes parasitized with *P. berghei* possessed a much higher hexokinase activity.

The inhibiting action of various compounds on the hexokinase of *P. berghei* closely resembled those with yeast hexokinase. Again all antimalarial compounds (apart from proguanil) inhibited the enzyme, but some of the most potent inhibitors were devoid of antimalarial action. Amongst the chemotherapeutically active compounds, there appeared to be an approximate parallelism between antimalarial activity and potency as inhibitors of plasmodial hexokinase. The action of mepacrine on plasmodial hexokinase was reduced by raising the concentration of ATP, but, as with yeast hexokinase, the inhibition by 10,732 was independent of the ATP concentration.

From a consideration of the results, it seems doubtful whether this type of inhibitory effect plays more than a minor part in the mechanism of antimalarial action *in vivo*.

It has been reported that certain antimalarial drugs, including mepacrine and quinine, have an inhibitory action on various enzymes of the avian malarial parasite *P. gallinaceum* (Speck and Evans, 1945 ; Marshall, 1948 ; Moulder, 1949). It has been suggested that the inhibitory action of these drugs on the enzymes of the parasite might be related to their chemotherapeutic activity, and further investigation appeared desirable. The present work has been mainly concerned with the enzyme hexokinase, on which the metabolism of glucose by the parasite primarily depends.

As experiments with human malarial parasites were not practicable, it appeared desirable to use some other mammalian parasite, rather than the avian plasmodium. *P. berghei,* which infects small rodents, including mice and rats, was an obvious choice. In order to determine the best conditions for performing the inhibition experiments, preliminary studies were carried out with a partially purified sample of yeast hexokinase. These results afforded an interesting comparison with the analogous results obtained later with the enzyme of the malarial parasite.

The main object of the work was to ascertain whether any connexion appeared to exist between the antimalarial activity of the drugs and their inhibitory action on the enzyme. It was therefore decided to include in the experiments a number of compounds devoid of antimalarial action so that their anti-hexokinase activity might be determined.

It was suggested by Bovarnick, Lindsay, and Hellerman (1946) that mepacrine might interfere with the phosphorylation of glucose in malarial parasites by competition with adenosinetriphosphate (ATP), and Work and Work (1948) pointed out that the possibility could not be excluded that mepacrine acted by combining with ATP. Experi-

*Present address: Department of Physiology, The Medical School, University of Birmingham.

ments were therefore carried out to investigate this possibility, and to elucidate the mechanism whereby certain of the drugs inhibited the enzyme.

Materials and Methods

Adenosinetriphosphate

ATP was isolated as the barium salt by the method of Dounce, Rothstein, Beyer, Meier, and Freer (1948). The barium ATP was converted into the potassium salt, a 5% excess of the theoretical amount of K_2SO_4 being used, and was stored at a final concentration of 0.05 M at −15° C.

Yeast Hexokinase

Hexokinase was prepared according to the procedure of Slater (1953) after autolysis of fresh baker's yeast by the method of Allfrey and King (1950). It was stored as a paste at −15° C., and retained its high activity over a period of three years. This active preparation was highly diluted before use, so that in any one experiment the concentration of $(NH_4)_2SO_4$ was negligible; therefore the preparation was not dialysed before use.

Plasmodial Hexokinase

A haemolysate of blood from mice infected with *P. berghei* was used as a source of hexokinase from the malarial parasite. The original strain of *P. berghei* was obtained from Dr. D. G. Davey, Imperial Chemical (Pharmaceuticals) Ltd. Young animals were more easily infected than those which were more than three months old, and so mice weighing 18–22 g. were used. They were inoculated intraperitoneally with 30 to 40 million red cells infected with the parasite, and were anaesthetized with chloroform about 3½ days later at the peak of parasitaemia. About 0.5 ml. blood was obtained from each mouse by cardiac puncture. Blood haemolysates were prepared by a slight modification of the method used by Speck and Evans (1945), who restored the salt concentration of the haemolysate to 0.9%. This was found to be unnecessary, and even inhibitory, and was omitted.

Antimalarial Drugs and Related Compounds

The compounds listed below were examined.

Number	*Chemical name*
5741	N^1-*o*-chlorophenyl-N^5-*iso*propylbiguanide HCl
5943	N^1-3 : 4-dichlorophenyl-N^5-*iso*propylbiguanide HCl
10,580	4 : 6-diamino-l-*p*-chlorophenyl-1 : 2-dihydro-2 : 2-dimethyl-1 : 3 : 5-triazine HCl
10,732	4 : 6-diamino-1-(3 : 4-dichlorophenyl)-1 : 2-dihydro-2 : 2-dimethyl-1 : 3 : 5-triazine HCl
5109	4-amino-6-*p*-chloroanilino-1 : 2-dihydro-2 : 2-dimethyl-1 : 3 : 5-triazine
15,587/B	4-amino-6-(3 : 4-dichloroanilino)-1 : 2-dihydro-2 : 2-dimethyl-1 : 3 : 5-triazine
6740/B	3-methoxy-8-chloro-5-(7-diethylaminoheptylamino)-acridine 2HCl
5068/B	2 : 3-dimethyl-4-(3-diethylaminopropylamino)-quinoline 2HCl

Determination of Hexokinase Activity

Method A.—The reaction mixture was buffered at *p*H 7.6 with aminotrishydroxymethylmethane (tris) (Gomori, 1946) or potassium phosphate with a final concentration of 0.0105 M, and normally contained: glucose, 0.00125 M; ATP, 0.004 M; $MgCl_2$, 0.005 M; drug, 0.004 M or distilled water. Diluted solution of stock yeast hexokinase preparation (0.1 ml.) or blood haemolysate (0.2 ml.) was added; the total volume was 1.2 ml. The enzyme underwent preliminary incubation with the drug for 15 min. at 20° C., or with one of its substrates and the drug at 30° C. The remaining substrates were then added, and the mixture was incubated for a further 15 min. at the same temperature as before. The reaction mixtures were deproteinized with $Ba(OH)_2$–$ZnSO_4$ reagents of Somogyi (1945), and glucose was determined in the filtrates by the copper reduction method of Nelson (1944).

Method B.—Hexokinase activity was measured in some experiments by the Warburg manometric technique. The system was buffered with $NaHCO_3$ in an atmosphere of N_2+CO_2 (95 : 5). At 37° C. under these conditions, a concentration of 0.02 M-$NaHCO_3$ is required to maintain a *p*H of 7.6. The glucose-6-phosphate produced in the hexokinase reaction released CO_2 from the bicarbonate, and the enzyme activity was measured by the production of CO_2 in µl. The reaction mixture normally contained: glucose, 0.005 M; ATP, 0.004 M; $MgCl_2$, 0.005 M; drug, 0.004 M; $NaHCO_3$, 0.02 M; and diluted stock yeast hexokinase preparation (0.2 ml.); the total volume was 1.2 ml. The flasks were equilibrated for 20 min. at 37° C., one of the substrates in water containing 0.02 M-$NaHCO_3$ being in the side-arm. This period also served as a preliminary incubation period for the enzyme-inhibitor complex. The reaction was started by tipping in the substrate from the side-arm and allowed to proceed for a further period (usually 30 min.), readings being taken at 5–10 min. intervals.

Determination of Inorganic Phosphate

Inorganic phosphate was determined by the method of Berenblum and Chain (1938) as modified by Long (1943).

Production of Reticulocytosis

Mice of 20 g. and rats of 100 g. were treated with acetyl phenylhydrazine for 8 to 10 days. They received a daily intraperitoneal injection of 0.2 ml. normal saline containing 2 mg./ml. acetyl phenylhydrazine for each 100 g. body weight. The blood of the animal was withdrawn by heart puncture 48 hr. after the last injection. Microscopic examination following staining with cresyl blue showed that, both in rats and mice, 70–80% of the red cells were present as reticulocytes.

Results

Stability of Partially Purified Yeast Hexokinase Preparations

The partially purified yeast hexokinase preparation which was used in this work underwent ther-

mal inactivation when incubated in the absence of its substrates at temperatures of 30° C. and over, and at a *p*H of 7.6. Since the experimental method involved the incubation of the enzyme with the drug before the start of the enzymic reaction, the degree of thermal inactivation of the enzyme during this preliminary period was investigated. The yeast hexokinase preparation in a dilution of 1:2,500 was incubated at 30° C. in phosphate buffer at *p*H 7.6 for 30 min., either alone or in the presence of ATP 0.004 M, glucose 0.00125 M, versene (ethylenediaminotetraacetic acid) 0.001 M, or cysteine 0.004 M. All four compounds increased the stability of the enzyme; the amount of glucose used in 15 min. was about three times as much as that used by the enzyme after incubating alone.

The thermal inactivation of the enzyme when incubated alone at 30° C. was lowest when the enzyme preparation was highly diluted, a dilution of 1:1,500 retaining considerably more activity than one of 1:200 and 1:600. The dilution after addition of substrates was adjusted to 1:2,500. Further, it was shown that at a final dilution of 1:3,000 the curve of enzyme activity against time was almost linear from 5 to 55 min., which suggests uniform enzyme activity during this period. At a dilution of 1:2,500 the enzyme did not lose its activity for periods up to 1 hr. on incubation at 20° C. when buffered to *p*H 7.6 with tris 0.0105 M or K phosphate buffer 0.0105 M (Table I). Some experiments on yeast hexokinase

TABLE I

EFFECT OF TEMPERATURE ON THE RATE OF INACTIVATION OF YEAST HEXOKINASE AFTER PRELIMINARY INCUBATION WITH TRIS AND WITH PHOSPHATE BUFFERS

Yeast hexokinase 0·1 ml. was first incubated with tris 0·0105M or phosphate 0·0105M *p*H 7·6, for various times at 20, 30, and 37° C. ATP 0·004M, glucose 0·00125M, and $MgCl_2$ 0·005M were then added; total vol. 1·2 ml. Incubated for 15 min. at 30° C. Final dilution stock yeast hexokinase preparation was 1: 2,500. Initially 1·5 μmole glucose was present in each sample.

Buffer	Preliminary Incubation Time (min.)	Glucose Used (μmoles)		
		20° C.	30° C.	37° C.
Tris ..	0	1·25	1·25	1·31
	15	1·36	1·06	0
	30	1·36	0·72	0
	60	1·42	0·53	0
Phosphate	0	1·23	1·25	1·28
	15	1·28	1·04	0
	30	1·35	0·80	0
	60	1·40	0·59	0

and all experiments on plasmodial hexokinase were carried out at 20° C. in the presence of tris or K phosphate buffer at *p*H 7.6. A considerable number of experiments on yeast hexokinase was carried out at 30° C. or 37° C. before the advantage of working at 20° C. had been appreciated.

The Inhibition of Yeast Hexokinase by Antimalarial Drugs and Related Compounds

Mepacrine, chloroquine, 10,580, 10,732, and quinine, all active antimalarial drugs, inhibited the action of yeast hexokinase to some extent, but not all inhibiting compounds were antimalarial drugs (Table II). Thus 5109, an isomer of 10,580, and 15,587/B, an isomer of 10,732, although they had

TABLE II

INHIBITION OF YEAST HEXOKINASE BY ANTIMALARIAL DRUGS AND RELATED COMPOUNDS

The numerals in brackets indicate the range of results. In method B, the results given are for preliminary incubation for 20 min. at 37° C. of (a) enzyme with drug and ATP: glucose in the side-arm, and of (b) drug with ATP: enzyme and glucose in side-arm.

Drug	Method A. Av. % Inhibition	No. of Expts.	Method B. Av. % Inhibition		
			(a)	No. of Expts.	(b)
Proguanil ..	0		0		—
6740/B ..	0		0		—
5741 ..	—		10 (—)	2	—
5068/B ..	—		16 (12–19)	6	—
Quinine ..	—		18 (13–20)	6	—
Mepacrine ..	18 (16–22)	6	23 (16–30)	8	8
10,580 ..	—		20 (18–23)	8	5
10,732 ..	20 (17–23)	6	22 (19–24)	8	5
Chloroquine	31 (30–33)	8	32 (30–37)	6	10
5109 ..	—		41 (36–44)	6	—
15,587/B ..	73 (—)	2	70 (65–73)	8	13

no antimalarial activity, had a very powerful inhibiting action. Proguanil had an effect on the estimation of glucose by reducing the amount of colour produced by the Nelson reagents. The difficulty was overcome by the use of appropriate controls containing proguanil, and it was tentatively concluded that proguanil was unable to inhibit yeast hexokinase. This conclusion was confirmed by results obtained with the Warburg manometric technique.

The Effect of Various Factors on the Degree of Inhibition of Yeast Hexokinase by Antimalarial Drugs and Related Compounds

Time of Addition of Drug.—The percentage inhibition was much less when the drug was in contact with ATP during the preliminary incubation period than when it was in contact with the enzyme during this period (Table III). Also, the degree of inhibition increased progressively, and was determined by the total time that the drug had been in contact with the enzyme, regardless of whether the drug was added at the beginning of the preliminary incubation or at some later stage in the reaction. These results suggested that the drug was acting primarily on the enzyme and not on ATP.

Concentration of ATP.—The degree of inhibition of yeast hexokinase by mepacrine and 10,732

TABLE III

EFFECT OF VARYING CONCENTRATIONS OF ATP ON THE INHIBITION OF YEAST HEXOKINASE BY MEPACRINE AND 10,732

Reaction medium: glucose 0·00125M; K phosphate buffer 0·01M, *p*H 7·6; $MgCl_2$ 0·005M; yeast hexokinase 0·1 ml.; total vol. 1·2 ml. Preliminary incubation for 15 min. at 30° C. with either glucose or ATP; reaction then allowed to proceed for 15 min. at 30° C. Final dilution stock yeast hexokinase preparation was 1: 2,500.

Drug	Final Concn. (M)	Concn. ATP (M)		% Inhibition
		During Preliminary Incubation	Added at Beginning Reaction	
Mepacrine	0·004	—	0·001	100
	0·004	—	0·002	88
	0·004	—	0·004	68
	0·004	—	0·008	25
	0·002	0·002	—	50
	0·004	0·002	—	76
	0·002	0·004	—	0
	0·004	0·004	—	0
10,732 ..	0·004	—	0·001	67
	0·004	—	0·002	69
	0·004	—	0·004	64
	0·004	—	0·008	61
	0·002	0·002	—	64
	0·002	0·004	—	64
	0·004	0·002	—	70
	0·004	0·004	—	74

in the presence of varying amounts of ATP is shown in Table III. When yeast hexokinase had undergone preliminary incubation in the presence of glucose and mepacrine, the inhibition produced was diminished if a large amount of ATP was added at the end of the preliminary incubation. However, when ATP and mepacrine, but not glucose, were present during the preliminary incubation the inhibition was not so great, and 0.004 M-ATP was sufficient to protect the enzyme completely. With 10,732 the percentage inhibition was almost independent of the concentration of ATP.

Concentration of Glucose.—When the concentration of glucose was raised from 0.00125 M to 0.0025 M there was no reduction in the inhibition by mepacrine. After preliminary incubation of yeast hexokinase for 15 min. at 30° C. with ATP 0.002 M and mepacrine 0.002 M and 0.004 M, glucose 0.00125 M or 0.0025 M was added. The inhibitory activity of mepacrine was no weaker at the higher concentration of glucose. However, in this type of experiment the amount of glucose could not be usefully increased to any great extent, because only a very small proportion of the total would then be utilized by the enzyme. In these circumstances, experimental errors in the determination of the percentage inhibition tend to become very great.

Further Studies at 20° C.—Certain of these results were confirmed at 20° C. (Table IV). At this temperature the enzyme does not undergo appreciable thermal inactivation within 3 hr., and so the action of inhibitors could be investigated in the absence of both glucose and ATP. With mepacrine at 20° C. the degree of inhibition increased when the preliminary incubation period was extended to 2 hr., but it tended to reach a limit after this time. The inhibitory power of mepacrine was again antagonized by ATP. On the other hand, the inhibitory potency of 10,732

TABLE IV

INHIBITORY EFFECT OF MEPACRINE, 10,732, AND 15,587/B ON YEAST HEXOKINASE AFTER INCUBATION FOR VARIOUS TIMES AT 20° C.

Reaction mixture: glucose 0·00125M; $MgCl_2$ 0·005M; K phosphate buffer 0·01M, *p*H 7·6; yeast hexokinase 0·1 ml.; total vol. 1·2 ml. Enzyme was first incubated with drug and phosphate at 20° C. Substrates were then added and the reaction proceeded for 7½ min. at 20° C. Final dilution of stock yeast hexokinase preparation was 1: 2,500.

		ATP	% Inhibition Observed on Preliminary Incubation for:				
			0	½	1	2	3 hr.
Mepacrine	0·001M	0·004M	0	9	18	26	26
,, ..	0·001M	0·002M	0	10	21	35	35
,, ..	0·002M	0·004M	0	26	26	41	41
,, ..	0·002M	0·002M	0	30	30	50	50
10,732 ..	0·001M	0·004M	0	13	22	22	22
,, ..	0·001M	0·002M	0	13	22	22	22
,, ..	0·002M	0·004M	0	13	22	38	47
,, ..	0·002M	0·002M	0	16	25	34	44
15,587/B	0·001M	0·004M	0	14	38	59	59
,, ..	0·001M	0·002M	17	24	45	69	69
,, ..	0·002M	0·002M	28	59	90	90	90

was not affected by ATP. With low concentrations of this drug the degree of inhibition increased during the period of contact with the enzyme up to 1 hr., but longer incubation resulted in no further inhibition. With higher concentrations of 10,732 inactivation was more rapid, and rose steadily over the whole period (3 hr.) in which observations were made. Similar results were obtained with 15,587/B, which proved to be a powerful inhibitor of yeast hexokinase. With this compound a decrease in enzyme inhibition resulted from an increase in the ATP concentration, suggesting some degree of competition between the drug and ATP. K fluoride 0.0035 M did not affect the uitilization of glucose by yeast hexokinase either in the presence or absence of drugs under the conditions in Table IV.

P. berghei *Hexokinase and Erythrocyte Lysates*

Adenosinetriphosphatase and Glucose-6-phosphatase.—The presence of adenosinetriphosphatase and glucose-6-phosphatase in haemolysates of normal and parasitized blood might introduce an error into the estimation of hexokinase activity. When normal mouse blood haemolysate was incubated with ATP or with glucose-6-phosphate, there was no increase in the inorganic phosphate

content. It was concluded that the activity of these enzymes was negligible. When a haemolysate of parasitized mouse erythrocytes was incubated with glucose-6-phosphate, there was again no detectable increase in inorganic phosphate ; but on incubation with ATP 0.004 M at *p*H 7.6 and at 20° C., 1.43 μg. inorganic phosphate was liberated presumably by the action of ATP-ase. This increase in inorganic phosphate was prevented by the incorporation of KF 0.0035 M in the haemolysate.

The Hexokinase Activity of Haemolysates of Normal and Parasitized Erythrocytes.—The hexokinase activity of normal and parasitized erythrocyte haemolysates was determined at 20° C. and *p*H 7.6 (Table V). Both rat and mouse erythrocytes were used in these experiments and gave

TABLE V

HEXOKINASE ACTIVITY OF HAEMOLYSATES OF NORMAL AND PARASITIZED ERYTHROCYTES AND OF RETICULOCYTES

Reaction mixture: glucose 0·00125M; KF 0·0035M; $MgCl_2$ 0·005M; ATP 0·004M; tris 0·0105M, *p*H 7·6; haemolysate 0·2 ml.; total vol. 1·2 ml. Incubation for 15 min. at 20° C.

Type of Haemolysate	Animal	Glucose Used (μmoles)
Normal	Mouse	0·14–0·20
„	Rat	0·12–0·26
70–80% parasitaemia	Mouse	0·80–1·20
70–80% „	Rat	0·84–1·40
70–80% reticulocytosis	Mouse	0·16–0·24
70–80% „	Rat	0·10–0·30

similar results. The hexokinase activity of the parasitized haemolysates was very much greater than that of a haemolysate of normal cells.

Certain malarial parasites, including *P. berghei*, show a strong predilection for reticulocytes, and in malarial infections there is a high degree of reticulocytosis. Sherwood Jones, Maegraith, and Gibson (1953) have shown that the respiration of rat reticulocytes is many times greater than that of mature red cells. The increased hexokinase activity of parasitized blood haemolysates as compared with normal haemolysates might therefore be related to the high degree of reticulocytosis rather than to the degree of parasitaemia. Table V shows that the hexokinase activity of blood haemolysates of animals with a high degree of reticulocytosis (70–80%) was not significantly higher than that of normal blood haemolysates. The high hexokinase activity of haemolysates of malarial erythrocytes is thus almost certainly due to the parasites themselves.

The glucose content of a haemolysate of highly parasitized blood was extremely low, and seldom exceeded 5–10 μg. in 0.2 ml. haemolysate, which is equivalent to 5 mg. corpuscular glucose/100 ml. whole blood as compared with an average value of 36 mg./100 ml. for whole normal blood. In many cases the glucose content of the parasitized haemolysate was so low that it could not be detected.

Stability and pH Optimum of Plasmodial Hexokinase.—The plasmodial hexokinase underwent no significant loss in activity when kept at 20° C. for 1 hr., 0.95 μmoles glucose being used by the fresh enzyme and 0.91 μmoles after storage. The plasmodial enzyme was observed to have a broad optimum from approximately *p*H 6.9–8.1.

The Inhibition of Plasmodial Hexokinase by Antimalarial Drugs and by Certain Other Compounds.—The compounds selected for study included certain antimalarial drugs, a number of related compounds, and also several alkaloids, primary aromatic amines, quinoline, and the powerful detergent cetrimide. An attempt was made to test 5943 and pyrimethamine, but these were very sparingly soluble at *p*H 7.6. The results were broadly parallel with those obtained using yeast hexokinase (Table VI). Mepacrine and

TABLE VI

INHIBITION OF PLASMODIAL HEXOKINASE BY ANTIMALARIAL DRUGS AND CERTAIN OTHER COMPOUNDS

Reaction medium: glucose 0·00125M; ATP 0·004M; $MgCl_2$ 0·005M; KF 0·0035M; tris 0·0105M, *p*H 7·6; inhibitor 0·004M; haemolysate 0·2 ml. Total vol. 1·2 ml. The haemolysate was incubated with buffer and drug for 15 min. at 20° C. Substrates were then added, followed by 15 min. incubation at 20° C.

Compound	% Inhibition
Proguanil	0
6740/B	0
5741	5
5068/B	9
Azacrin	11
5109	20
Quinine	20
10,580	22
Chloroquine	26
10,732	29
Mepacrine	32·5
15,587/B	60
Aniline	0
o-Toluidine	0
Quinoline	11
Brucine	13
Morphine	15
Strychnine	30
Cetrimide	100

10,732 appeared to be relatively more powerful inhibitors of the plasmodial enzyme than of the yeast enzyme ; chloroquine was more active than either of these against yeast hexokinase. Strychnine was as potent an inhibitor of plasmodial hexokinase as mepacrine. Cetrimide proved to be the most powerful inhibitor of the enzyme. At a concentration of 0.002 M and under the conditions shown in Table VI, it inhibited plasmodial hexokinase to the extent of 50%.

The Effect of the Concentration of ATP on the Inhibition of Plasmodial Hexokinase by Mepacrine and 10,732.—From the results in Table VII it appeared that the inhibition of plasmodial hexokinase by mepacrine was the higher the lower the

TABLE VII

EFFECT OF THE CONCENTRATION OF ATP ON THE INHIBITION OF PLASMODIAL HEXOKINASE BY MEPACRINE AND 10,732

Conditions as in Table VI

ATP (Final Concn. M)	% Inhibition Mepacrine	% Inhibition 10,732
0·004	30	30
0·002	40	29
0·001	57	35

concentration of ATP, but with 10,732 the inhibition was not influenced by ATP in this manner. The results closely resembled those obtained with yeast hexokinase.

DISCUSSION

The results presented indicate that the drugs inhibit yeast hexokinase by attacking the enzyme itself, rather than by combining with ATP. The degree of inhibition increases as the time during which the enzyme has been in contact with the drug is lengthened. The higher the concentration of ATP, the lower is the degree of inhibition. The inhibitory action of the drug, however, appears to be independent of the concentration of glucose.

These results can be partially explained if it is assumed that there are two types of active groups on the enzyme surface. Those of the first type are glucose acceptors. Glucose is not so effective at protecting yeast hexokinase as ATP, and, since the degree of inhibition appears to be independent of the glucose concentration, the mepacrine molecules presumably do not interfere at these sites. The active groups of the second type are ATP acceptors. When there is no ATP present during the preliminary incubation period the mepacrine molecules are able to occupy these sites to the exclusion of ATP, and inhibition results. If much ATP is added at the end of the preliminary incubation period, the degree of inhibition is reduced; this suggests that ATP can displace mepacrine from the sites, and so antagonize its action. If, on the other hand, the ATP is present during the preliminary incubation of the enzyme with mepacrine, fewer of the ATP acceptors are available for mepacrine and the resulting inhibition is less. The progressive nature of the inhibition may be due to a slow local modification of the structure of the hexokinase molecule when the ATP acceptors are occupied by mepacrine. This structural change may perhaps be regarded as a local denaturation of the protein. The ATP, on displacing the mepacrine, may be able to reverse this "denaturation" provided that it has not gone too far, and thus restore the original structure of the yeast hexokinase.

The results in Table V demonstrate that, in spite of the high respiration rate of rat and mouse reticulocytes compared with normal erythrocytes (Sherwood Jones *et al.*, 1953), there is no corresponding difference in hexokinase activity. Calculation shows that if all the observed oxygen uptake of the reticulocyte depends on the oxidation of glucose, and if this glucose is first phosphorylated under the influence of hexokinase, then the hexokinase present is amply sufficient to account for the respiration of the cells. Thus there is no inconsistency between the hexokinase results and those reported by Sherwood Jones *et al.* on the rate of oxygen uptake by reticulocytes. It would appear that the hexokinase activity of the reticulocytes remains almost intact in the mature erythrocyte, along with the rest of the activity of the glycolytic system, whereas the tricarboxylic acid cycle no longer functions. The oxygen uptake of the mature erythrocyte is therefore very small or non-existent.

It may be argued that it is preferable to separate the parasites from the haemolysate, so that the parasite enzymes may be studied without contamination with those of the erythrocytes. This, however, involves the use of a haemolytic agent such as saponin, and it appears advantageous, in the first instance at least, to adopt the simpler method of extracting the parasitized erythrocytes with distilled water, and thus avoid the use of surface active agents. The hexokinase activity of both normal erythrocytes and reticulocytes has been shown to be very small compared with that of the parasites. Any contribution in the haemolysates of parasitized cells made by the erythrocytes would presumably be significantly less than would be possessed by haemolysates of a comparable number of normal cells, in consequence of the physical presence of the parasites within the former.

It appears from Table II that all active antimalarial drugs inhibit yeast hexokinase, but certain compounds devoid of antimalarial activity have also an inhibitory effect. Tables II and VI show that there is close similarity between the results obtained with yeast hexokinase and those with the plasmodial enzyme.

The information on the chemotherapeutic action of the compounds against *P. berghei* is somewhat conflicting. From the review by Thurston (1953)

it appears that the most potent plasmodicidal effect is produced by pyrimethamine, which it was unfortunately impossible to investigate in the present work because of the very low solubility of it and its salts.

Though not so potent as mepacrine and chloroquine, proguanil is an effective agent against *P. berghei*, but has no inhibitory effect on hexokinase. It is generally agreed that proguanil itself has no significant antimalarial action, but is converted into the triazine, 10,580. This compound has a definite action on the enzyme, though less than that of mepacrine and chloroquine. This is in harmony with the weaker chemotherapeutic effect of proguanil compared with that of mepacrine or chloroquine, which are almost equally active against *P. berghei*. Unfortunately, there appears to be no information as to the relative efficacy of proguanil and its triazine metabolite against *P. berghei* in mice. Crowther and Levi (1953) found that 10,580 was 10 times as active as proguanil in suppressing *P. gallinaceum* infections in chicks. *P. berghei* is, however, less sensitive than *P. gallinaceum* to the action of proguanil. The results with the biguanyl compound, 5943, and its active triazine metabolite, 10,732, are in general agreement with these findings. Like proguanil, 5943 appeared to have little or no action on hexokinase, but precise results could not be obtained because of the very low solubility of the drug. It is, however, more potent as an antimalarial agent than proguanil, being approximately equal to mepacrine and chloroquine in its activity against *P. berghei*. In accordance with this, its metabolite, 10,732, has about the same anti-hexokinase activity as the latter two drugs.

Quinine has a very low antimalarial action against *P. berghei*, compared with mepacrine, but its inhibitory action on plasmodial hexokinase is about 20%, as compared with 32% for mepacrine under similar conditions. The chloroquine analogue, 5068/B, has only very slight antimalarial activity, which agrees well with its low inhibitory potency, and 6740/B, which completely failed to inhibit, is without antimalarial activity.

Thus it appears that all active antimalarial drugs tested, with the exception of proguanil, did inhibit plasmodial hexokinase, and there was at least an approximate correlation between their antimalarial activity and their inhibitory power. However, all inhibiting compounds were not chemotherapeutically active. Of the compounds related chemically to the series of antimalarial drugs, the most powerful inhibitor was 15,587/B, which is said to be completely inactive against avian malaria. 5109, which is also inactive, was as good an inhibitor as its active isomer, 10,580. Amongst other compounds tested on the hexokinase of *P. berghei*, several possessed appreciable inhibitory activity, including the alkaloids brucine, morphine, and strychnine. In view of the inhibition by these alkaloids, it is perhaps not surprising that quinine has an inhibitory action on plasmodial hexokinase higher than would be expected on the basis of its antimalarial activity. It is noteworthy that cetrimide had a higher anti-hexokinase activity than any other compound tested.

The inhibition of the enzyme by compounds devoid of antimalarial activity casts grave doubts on the validity of any theory of antimalarial action based on the inhibition of plasmodial hexokinase. It has always to be borne in mind, however, that many factors may deprive a compound of chemotherapeutic action *in vivo*, even although it may possess some of the essential properties for activity. There may be differences in the permeability of the erythrocyte membrane to the compound, or some mechanism, at present unknown, may render them inactive *in vivo*.

It is important to note that the concentrations of drugs required to produce an appreciable degree of inhibition are much higher than those required to bring about definite chemotherapeutic effects. This is a further argument against the view that the inhibition of the hexokinase system plays an important part in the action of antimalarial drugs. Nevertheless, in the intact animal, the drugs may be concentrated, either locally in various tissues, or in particular structures inside the cells, so that at those key sites the concentration of the drug may be very high.

It is possible that a really effective chemotherapeutic agent may exert several effects on parasite metabolism, some of which may be more essential than others, but which are complementary in destroying the organism. The antagonism of certain antimalarial agents to folinic acid may play a key role in establishing chemotherapeutic activity, but other functions, of which the anti-hexokinase activity may be one, may possibly fulfil a subsidiary role.

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References

Allfrey, V. G., and King, C. G. (1950). *J. biol. Chem.*, **182**, 367.

Berenblum, I., and Chain, E. (1938). *Biochem. J.*, **32**, 295.

Bovarnick, M. R., Lindsay, A., and Hellerman, L. (1946). *J. biol. Chem.*, **163**, 523, 535.

Crowther, A. F., and Levi, A. A. (1953). *Brit. J. Pharmacol.*, **8**, 93.

Dounce, A. L., Rothstein, A., Beyer, G. T., Meier, R., and Freer, R. M. (1948). *J. biol. Chem.*, **174**, 361.

Gomori, G. (1946). *Proc. Soc. exp. Biol., N.Y.*, **62**, 33.

Long, C. (1943). *Biochem. J.*, **37**, 215.

Marshall, P. B. (1948). *Brit. J. Pharmacol.*, **3**, 1.

Moulder, J. W. (1949). *J. infect. Dis.*, **85**, 195.

Nelson, N. (1944). *J. biol. Chem.*, **153**, 375.

Sherwood Jones, E., Maegraith, B. G., and Gibson, Q. H. (1953). *Ann. trop. Med. Parasit.*, **47**, 431.

Slater, E. C. (1953). *Biochem. J.*, **53**, 157.

Sols, A., and Crane, R. K. (1954). *J. biol. Chem.*, **206**, 927.

Somogyi, M. (1945). Ibid., **160**, 69.

Speck, J. F., and Evans, E. A. (1945). Ibid., **159**, 71, 83.

Thurston, J. P. (1953). *Exp. Parasitol.*, **2**, 311.

Work, T. S., and Work, E. (1948). *The Basis of Chemotherapy*. Edinburgh: Oliver and Boyd.