therapeutic dose and involving an amount of dextran sulphate corresponding to two or three days of therapy.

SUMMARY

1. Dextran [^{35}S]sulphate with molecular-weight distribution and biological properties similar to that used as a clinical anticoagulant has been prepared.

2. Intravenous injection of dextran sulphate is followed by renal excretion in exponentially decreasing amount. About 30% is eliminated in 2 days rising to 50% at 4 days.

3. Initially the excreted dextran sulphate is a low-molecular-weight component of the injected material.

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Inhibitors and Activators of Brain Hexokinase

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Inhibitions and activations of hexokinase by specific proteins are of great interest because of their possible connexion with the mechanism of hormonal action. Colowick, Cori & Slein (1947) found that a protein fraction of the anterior pituitary inhibited the hexokinase activity of brain and muscle extracts and that the inhibition was enhanced by adrenocortical extract and counteracted by insulin. Attempts to confirm this result have not always been successful. Abood & Gerard (1951) have reported that a protein in nerve extracts specifically inhibits brain hexokinase; inhibitors of hexokinase have also been found in extracts of spleen (Broh-Kahn & Mirsky, 1947), pancreas (Zakharyn, 1951) and placenta (Gerrard, private communication). Weil-Malherbe & Bone (1951a-d) made a detailed study of brain hexokinase and discovered activators of the enzyme in erythrocytes and muscle, and inhibitors and activators in human plasma. Zacco & Sevag (1952) have published a preliminary report on an inhibitor in human plasma. Further experiments with inhibitors and activators of hexokinase are described in this paper.

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EXPERIMENTAL

The experimental arrangements, analytical methods and composition of the medium for the assay of hexokinase activity were as used by Weil-Malherbe & Bone (1951a). Hexokinase activity was determined by measuring glucose disappearance (Nelson, 1944). Because of the high adenosinetriphosphatase (ATPase) activity of many of the preparations used, it was usually found convenient to make up the test medium in the following order and final concentrations (M): water; glucose, 0.00185–0.002; MgCl_2 , 0.0067; NaHCO_3 or KHCO_3 , 0.02; activators or inhibitors; NaF , 0.024. The reaction was started by the addition of adenosine triphosphate (ATP) to give a final concentration of 0.0033–0.005 M. Incubations were usually carried out at 30° for 15–20 min. in an atmosphere of N_2 containing 5% (by vol.) CO_2 . Any deviations from these conditions are mentioned in the text.

Brain extracts. Most of the experiments were carried out with an extract prepared by double extraction of rat brain at 0° with glass-distilled water and centrifuging in an angle centrifuge at 3500 rev./min. for 5 min. (Weil-Malherbe & Bone, 1951a). This preparation will be referred to as 'standard brain extract'. Some brain extracts were fractionated by centrifuging at 20 000 g for 20 min. in a refrigerated centrifuge, a procedure which practically eliminates particulate constituents other than microsomes. The supernatants will be referred to as high-speed extracts.

Plasma inhibitor. Blood samples (5–10 ml.) were obtained by venipuncture and delivered into bottles containing solid NaF as anticoagulant to give a final concentration of 0.3% (w/v). The NaF was dissolved by gently inverting the bottle for a few minutes and the plasma separated by centrifuging. The plasma was either used at once or exhaustively dialysed at 3° against running glass-distilled water.

Nerve suspensions. Sciatic and brachial nerves of male white rats were ground with sand and glass-distilled water at 0°. The mixture was centrifuged lightly to remove sand and tissue debris and the residue extracted once more with water. The aqueous suspension (1:20) was used without further purification.

Placental extracts. Aqueous extracts of human placenta (1:20) were made available through the courtesy of Dr J. W. Gerrard. The extracts were frozen by solid CO₂ and despatched in boxes containing solid CO₂. They were still frozen when received.

Erythrocyte lysates and rat-muscle extracts. Haemolysates and muscle extracts were prepared according to Weil-Malherbe & Bone (1951b). The muscle extracts were dialysed against running glass-distilled water at 3° and used without further purification.

In experiments involving activations or inhibitions, deviations $\geq 17\%$ from the control experiment were regarded as significant. Weil-Malherbe & Bone (1951d), using identical experimental methods, established that deviations $\geq 17\%$ were statistically significant at the 1% level.

RESULTS

Inhibitor in plasma

Occurrence. Significant inhibitions were found in four out of thirty-eight non-dialysed samples of plasma, i.e. in 10.5% of cases. Zacco & Sevag (1952) found inhibitions in 14% of their cases and Weil-Malherbe & Bone (1951d) in 13% of a series which included about 400 samples. In agreement with observations by Zacco & Sevag (1952), it was found that dialysis of plasma against glass-distilled water enhanced and stabilized the inhibitor activity. When the standard test medium contained 1–1.5 ml. of plasma, twenty-one out of twenty-four samples of dialysed plasma significantly inhibited brain hexokinase activity, in fourteen cases by over 30%. When blood samples were drawn at 30 min. intervals during a glucose-tolerance test, and the plasma was separated, dialysed and tested, the inhibitions observed did not vary significantly from sample to sample, nor were there any marked differences in inhibition among plasma samples from the same donor taken at intervals of several months.

Properties of the dialysed plasma inhibitor. The inhibitor was non-dialysable and relatively heat stable. The inhibition was not counteracted by insulin or reduced glutathione (GSH) (Table 1). The relation between inhibition and inhibitor concentration was non-linear; increasing the volume of

dialysed plasma beyond 1.5 ml./3 ml. reaction mixture did not appear to lead to further increase in inhibition (Fig. 1). Inhibition was maximal from the start of the reaction (Fig. 2); preincubation of the brain extract with plasma for 20 min. at 30° had no effect on the inhibition (Table 1). The activity of samples of non-dialysed plasma showing inhibition disappeared so quickly even at 3° that a systematic investigation of their properties was not practicable.

Specificity. Inhibitions were most consistently obtained with brain hexokinase and were about the same for standard and high-speed extracts (Table 2). Yeast hexokinase and brain phosphohexokinase were also inhibited, apparently to a somewhat smaller extent. Inhibition was also observed with fructose as substrate (Table 3).

Reversibility. It was found that the inhibition by dialysed plasma was not counteracted by salt addition. When KCl was added to plasma, which had been exhaustively dialysed against glass-distilled water, to give a final concentration of 0.67 M, the inhibition was not affected (Table 4).

Table 1. *Inhibition of brain hexokinase by dialysed plasma*

Standard brain extract, 0.3 ml.; dialysed plasma, 1.5 ml.; ATP, 15 μ moles; total vol., 3 ml. Other components in standard concentration.

Additions	Hexokinase activity (control = 100)
Plasma	65
Plasma + 0.5 mg. insulin	63
Plasma heated for 3 min. at 100°	65
Plasma heated for 10 min. at 55°	73
Plasma + 0.003 M GSH	63
Plasma preincubated with enzyme for 20 min. at 30°	66

Fig. 1. Effect of plasma concentration on the activity of rat-brain hexokinase. Brain extract, 0.3 ml.; ATP, 15 μ moles; total vol., 3 ml.; other constituents in standard concentration. Each symbol represents a different standard brain extract.

Mode of action. The inhibition by plasma is not due to ATPase or phosphatase activity. When the magnesium and ATP concentrations were increased fourfold the inhibition was not relieved. The Mg:ATP ratio was kept constant, as Hers (1952) has shown that the activity of brain hexokinase is reduced at Mg:ATP ratios below 1. Furthermore, when ATP was estimated at the end of the incubation by determining the acid-labile phosphate, corrected for hexose diphosphate (HDP) hydrolysis (Weil-Malherbe & Bone, 1951a), the ATP concen-

Fig. 2. Disappearance of glucose from rat-brain extract in the presence and absence of plasma inhibitor. Brain extract, 0.3 ml.; glucose, 6.66 μ moles; ATP, 15 μ moles; total vol., 3 ml. Curve A, brain extract; curve B, brain extract + 2 ml. dialysed plasma.

Table 2. *The effect of dialysed plasma on standard and high-speed extracts of brain*

Standard brain extract, 0.3 ml., or high-speed extract, 0.6 ml.; ATP, 15 μ moles; dialysed plasma, 2.0 ml. Other components in standard concentration.

Expt. no.	Type of extract	Hexokinase activity (control=100)
1	Standard	79
	High-speed	80
2	Standard	78
	High-speed	78

Table 3. *Specificity of plasma inhibitor*

Standard brain extract, 0.3 ml., or yeast hexokinase, 0.4 mg.; dialysed plasma, 1.5 ml.; ATP, 15 μ moles; other components in standard concentration; hexose monophosphate (HMP) in phosphohexokinase experiment, 15 μ moles; total vol., 3 ml. Phosphohexokinase activity measured by HDP formed.

No. of measurements	Enzyme	Substrate	Mean inhibition of enzyme activity (%)
5	Brain hexokinase	Glucose	38
5	Brain phosphohexokinase	HMP	22
2	Brain hexokinase	Fructose	20
2	Yeast hexokinase	Glucose	17

Table 4. *The effect of KCl on the inhibition of brain hexokinase by dialysed plasma*

Brain extract, 0.3 ml.; ATP, 15 μ moles; dialysed plasma, 1.7 ml. Other components in standard concentration.

Expt. no.	Additions	Hexokinase activity (control=100)
1	0.67 M-KCl	64
	Plasma	76
	Plasma + 0.67 M-KCl	49 (77*)
2	0.67 M-KCl	62
	Plasma	76
	Plasma + 0.67 M-KCl	48 (77*)

* Calculated for KCl-containing medium as control.

Table 5. *ATP concentration in brain extracts after 20 min. incubation*

Standard brain extract, 0.3 ml.; plasma, 1.5 ml. in Expt. 1, 1.8 ml. in Expt. 2; ATP, 15 μ moles; total vol. 3 ml.; other components in standard concentration.

Expt. no.	Additions	ATP found (μ moles)	Inhibition of hexokinase reaction (%)
1	Nil	6.4	—
	Plasma	7.0	45
2	Nil	7.1	—
	Plasma	8.6	47

tration in the presence of plasma was actually higher than in the control (Table 5). The inhibition was also unaffected when the glucose concentration was reduced by a factor of 4 and that of brain extract to 0.1 ml./3 ml. reaction mixture. At this, the lowest substrate concentration at which the inhibition could be accurately measured, the reaction was still of zero order with respect to glucose.

Experiments in which the inhibition of hexokinase is determined by measuring glucose disappearance are vitiated if reducing substances are produced in the reaction mixture (Stadie, Haugaard & Hills, 1950). This source of error may be excluded, as no reducing substances were produced when brain extracts were incubated for 20 min. at 30° in reaction mixtures containing inhibitor but no ATP. The results reported here suggest that the inhibitor acts directly on the enzyme.

Inhibitor in nerve and placenta

The necessity for showing, as in the preceding section, that inhibition of hexokinase is not caused by ATPase or phosphatase activity is well illustrated by the following experiments with nerve suspensions and placental extracts.

Suspensions from the nerves of twenty-two rats were tested. Of these, seven significantly inhibited brain hexokinase, but the effect per mg. of nerve was only 0.5–0.3 as great as that reported by Abood & Gerard (1951). The inhibitor lost most of its activity within a few hours, even at 3°. It could not be stabilized by dialysis against glass-distilled water. The inhibition by nerve suspensions could be counteracted by excess ATP (Fig. 3). This suggests that the inhibition is caused by ATPase activity. In fact, during incubation for 20 min. in the presence of fluoride, active preparations hydrolysed over 25% of the ATP in the reaction mixture, and ATPase activity of this order was always present when inhibition was observed. Phosphate formation from hexose monophosphate (HMP) and HDP was about 20 times smaller. Furthermore, it was found that incubation at 37° for 40 min., the experimental conditions of Abood & Gerard (1951), required a minimum ATP concentration for maximum activity of brain hexokinase of 0.006M, while these authors used 0.003M-ATP. At low initial ATP concentrations hydrolysis of ATP by ATPase will have an inhibitory effect on glucose phosphorylation and will also favour the accumulation of inhibitory HMP. The inhibitions observed by Abood & Gerard (1951) can be fully accounted for on this basis.

The inhibition of brain hexokinase by placental extracts proceeds by a similar mechanism. Inhibitions were consistently obtained both with crude extracts and with an ammonium sulphate fraction precipitated between 0.25 and 0.33 saturation. Again the inhibitions were reversible by excess ATP (Fig. 4). The placental extracts differed from the nerve suspensions in their ability to hydrolyse HMP and HDP as well as ATP.

Activators of hexokinase

Apparent activation of the hexokinase reaction by phosphohexokinase. In a recent paper on brain hexokinase, Crane & Sols (1953) found that the bulk of the enzyme sediments at 3500 g. The rate of glucose utilization by the sediment decreased with time as the phosphohexokinase (phosphohexose isomerase + phosphofructokinase) concentration in the mixture was insufficient to prevent the accumulation of inhibitory glucose 6-phosphate. The addition of phosphohexokinase led to a significant increase in the rate of glucose utilization.

Apparent activations of this type were observed in some cases with brain preparations which contained a much higher proportion of particulate hexokinase than the standard extract (Table 6). In the standard extract about 0.5–0.7 of the hexokinase activity was associated with particulate matter. The proportion of hexokinase left in the supernatant after centrifuging at 20000 g was thus considerably greater than that found by Crane & Sols (1953). This was also the case when isotonic

Fig. 3. Disappearance of glucose from rat-brain extract in the presence and absence of nerve suspension. Brain extract, 0.25 ml.; glucose, 7.8 μ moles; ATP, 7.5 μ moles; other constituents in standard concentration; total vol., 3 ml. Curve A, brain extract; curve B, brain extract + nerve suspension from 30 mg. nerve; curve C, same as curve B, with 5 μ moles ATP added at 30 min.

Fig. 4. Disappearance of glucose from rat-brain extract in the presence and absence of placental extract. Brain extract, 0.2 ml.; glucose, 5.55 μ moles; ATP, 10 μ moles; other constituents in standard concentration; total vol., 3 ml. Curve A, brain extract; curve B, brain extract + placental extract (10 mg. protein); curve C, same as curve B, with 10 μ moles ATP added at 30 min.

mannitol was used as suspending medium. The difference might be due to the use of a tissue grinder of closer fit. The utilization of HMP by the standard extract was about 2-3 times as great as that of glucose for a substrate concentration of 0.002M.

Activator in erythrocytes and muscle. The activation of brain hexokinase by extracts of erythrocytes and muscle (Weil-Malherbe & Bone, 1951b, c) cannot be explained by the HMP-phosphohexokinase mechanism. This was shown kinetically by carrying out an experiment at two concentrations of brain hexokinase. At the lower concentration it will take longer before an inhibitory concentration of HMP is produced, and if the activation is due to the removal of HMP by phosphohexokinase it should be greater at the higher concentration of hexokinase (Crane & Sols, 1953). It was found, however, that the reaction was linear at two con-

centrations of brain hexokinase, namely 0.3 and 1.0 ml. of a high-speed extract in a total volume of 3 ml., and the activation was the same in both cases (Fig. 5). This result, together with the findings of Weil-Malherbe & Bone (1951b, c), that haemolysates and muscle extracts in which phosphohexokinase activity had been destroyed by heat were still able to activate brain hexokinase, provide conclusive evidence for the non-identity of the activator and phosphohexokinase.

Effect of salt concentration on the activation of brain hexokinase. When standard brain extract was incubated in the presence of 0.67M-KCl or 0.003M-CaCl₂ and activator, hexokinase activity was somewhat reduced but the activation was completely abolished (Table 7).

Effect of brain preparation on activation. One muscle extract was tested with a series of brain extracts. 1 ml. of the muscle extract formed 0.44 μ mole of HDP from 0.005M-HMP in 20 min. under the conditions of the standard test. The phosphohexokinase activity of 1 ml. of brain extract was 20-30 times greater. Increasing the volume of muscle extract added beyond 0.3 ml. in 3 ml. reaction mixture did not lead to further increase in activation. It was found in these experiments that the extent of the activation varied with different brain extracts. Furthermore, it was sometimes the high-speed extract and sometimes the standard extract which was more susceptible to activation (Table 8). This strongly suggests that the activation depends on a factor in the brain preparation.

Fig. 5. Activating effect of dialysed rat-muscle extract on brain hexokinase at two concentrations of brain extract. High-speed extract, 0.3 ml. (x) and 1.0 ml. (O) in 3 ml. total volume; muscle extract, 1.0 ml. Curve A, brain extract; curve B, brain extract + muscle extract. Glucose disappearance expressed in mg./ml. of brain extract used. Both curves corrected for the hexokinase activity of the muscle extract.

DISCUSSION

In the experiments with nerve and placental extracts the observed inhibitions could not be separated from the ATPase or phosphatase activity of the preparations. On the other hand, the data of this paper confirm the existence of an inhibitor of

Table 6. *Hexokinase activity of brain preparations separated by fractional centrifuging*

Rat brain was macerated in 6 ml. 5.75% mannitol, centrifuged 5 min. at low speed and 20 min. at high speed. Both sediments were washed once with mannitol, recentrifuged, and made up to original volume; 0.2 ml. was added to standard test system.

Expt. no.	Fraction	Centrifugal force (g)	Disappearance of glucose (mg.)	Apparent activation*
1	A (Sediment)	1 500	0.295	—
	B (Sediment)	20 000	0.137	—
	C (Supernatant)	20 000	0.096	—
	B + C	—	0.268	115
	A + C	—	0.844	216
2	A (Sediment)	1 200	0.263	—
	B (Sediment)	20 000	0.251	—
	C (Supernatant)	20 000	0.117	—
	A + B + C	—	0.617	98

* Apparent activation = ratio of activity of combined fractions to sum of activities of individual fractions tested separately, $\times 100$.

Table 7. *The effect of electrolyte concentration on the activation of brain hexokinase by haemolysates*

Haemolysate heated 5 min. at 80°; 1 ml. haemolysate added to standard test.

Expt. no.	Additions	Hexokinase activity (control = 100)
1	0.85 M-KCl	57
	Haemolysate	140
	Haemolysate + 0.85 M-KCl	54
2	0.67 M-KCl	72
	Haemolysate	146
	Haemolysate + 0.67 M-KCl	63
3	0.003 M-CaCl ₂	59
	Haemolysate	129
	Haemolysate + 0.003 M-CaCl ₂	63

Table 8. *The effect of a rat-muscle preparation on a series of brain extracts*

Standard brain extract, 0.3 ml.; high-speed extract, 1.0 ml.; ATP, 15 μmoles. Other components in standard concentration.

Brain extract	Type of extract	Muscle extract (ml.)	Hexokinase activity of brain extract* (control = 100)
1	Standard	0.3	160
	High-speed	0.6	115
3	Standard	0.6	159
	High-speed	0.6	153
4	Standard	1.0	125
	High-speed	1.0	152
5	Standard	1.0	127
	High-speed	1.0	154
	Standard	0.7	130
	High-speed	0.7	159

* Corrected for the hexokinase activity of the rat-muscle extract.

hexokinase in plasma and provide conclusive evidence that the inhibition is not due to phosphatase or ATPase activity. With dialysed plasma, inhibitions are observed in a high proportion of cases. The mechanism by which dialysis enhances and stabilizes the inhibition is not clear. There can be little doubt that the inhibitor in dialysed plasma is identical with that described by Zacco & Sevag (1952). There are, however, important differences in the properties of the inhibitor in dialysed plasma and the inhibitor in fresh plasma described by Weil-Malherbe & Bone (1951*d*). The latter is less stable, occurs less regularly, and the magnitude of the inhibition shows much greater fluctuations over the 3 hr. period of a glucose-tolerance test. Weil-Malherbe & Bone (1951*d*) also found that the inhibitions produced by fresh plasma could be partially relieved by insulin, which was found to have no effect on the inhibition by dialysed plasma.

There is evidence from the work of Christensen, Plimpton & Ball (1949) that an inhibitor of erythrocyte hexokinase is present in the plasma of normal, alloxan-diabetic and hypophysectomized rats. Very recently, Bornstein & Park (1953) and Bornstein (1953) have reported that the β-lipo-protein fraction of the serum of alloxan-diabetic rats inhibits the glucose uptake of rat diaphragm. The inhibitor, which is antagonized by insulin, appears to act at the hexokinase level. This inhibitor was not detected in the serum of normal rats or the serum of diabetic rats which had in addition been hypophysectomized or adrenalectomized. There exists, therefore, the possibility that human plasma and rat serum contain more than one inhibitor of hexokinase.

The significant feature of the experiments with muscle extracts and haemolysates is that the observed activations cannot be accounted for by phosphohexokinase activity. In this connexion it may be mentioned that an activator of hexokinase, free from hexokinase and phosphohexokinase activity, has recently been prepared from ox brain (Weil-Malherbe, private communication). The fact that the activation is abolished by salt is also difficult to reconcile with the view that phosphohexokinase is responsible for the activation. Furthermore, it has been shown that the activation depends on the hexokinase preparation as well as the activator. It appears that the simplest explanation of the experiments with activators is that brain preparations contain an inhibitor. Evidence of a similar kind is provided by the observations of Weil-Malherbe & Bone (1951*d*) that some samples of fresh plasma gave highly significant activations with some and highly significant inhibitions with other preparations of hexokinase. It seems plausible therefore, to visualize the activation process in terms of a competition of specific proteins for an inhibitor associated with hexokinase.

SUMMARY

1. The presence in plasma of an inhibitor of hexokinase has been confirmed. Dialysis against glass-distilled water stabilizes and enhances the inhibition. The inhibitor is non-dialysable, fairly heat stable, and not counteracted by insulin. Brain hexokinase with glucose as substrate is most susceptible to inhibition, but the phosphorylations of fructose by brain hexokinase, of hexose monophosphate by brain phosphohexokinase, and of glucose by yeast hexokinase are also inhibited.
2. The inhibition by dialysed plasma is not due to adenosinetriphosphatase activity and is not affected by a fourfold change in the concentration of magnesium, adenosine triphosphate or glucose. It is

suggested that the inhibitor acts directly on the enzyme.

3. The inhibitions of brain hexokinase by nerve and placenta is fully accounted for by their adenosine triphosphatase and phosphatase activities.

4. The activation of hexokinase by proteins in muscle and haemolysates has been confirmed. The phenomenon cannot be adequately explained by the removal of hexose monophosphate by phosphohexokinase. The activation is abolished by 0.67M-KCl and 0.003M-CaCl₂. Evidence is given that the activation depends on the hexokinase preparation.

5. The relation of the plasma inhibitor to similar factors described in the literature is discussed. It is tentatively suggested that the activation process involves the competition of specific proteins in the activator preparation for an inhibitor associated with hexokinase.

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Metabolism of Dehydroascorbic Acid and the Effect of Adrenocorticotrophic Hormone and Cortisone

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In studies designed to elucidate the problem of the fate of dehydroascorbic acid in guinea pigs, Gould & Shwachman (1943) found that oral or injected solutions of ascorbic acid, which had undergone oxidation by treatment with iodine, exerted an antiscorbutic effect. Penney & Zilva (1943) and Damron, Monier & Roe (1952), using solutions of ascorbic acid oxidized by potassium iodate and bromine respectively, found that single injections of dehydroascorbic acid were partly reduced to ascorbic acid and partly oxidized to diketogulonic acid.

To human subjects Johnson & Zilva (1934) gave oral solutions of dehydroascorbic acid prepared by iodine and hydrogen peroxide oxidation of lemon juice; Todhunter, McMillan & Ehmke (1950) administered orally solutions containing dehydroascorbic acid prepared by charcoal treatment of orange juice, while de Ritter, Cohen & Rubin (1951) gave dehydroascorbic acid or its methanolate by mouth. From the effects on plasma concentration

or urinary output or both, these investigators concluded that oral dehydroascorbic acid has the same effect as ascorbic acid. We are not aware of any experiments in which dehydroascorbic acid has been injected into human subjects.

Additional interest in dehydroascorbic acid was stimulated by the suggestion of Long, Miles & Perry (1951) based on the modification of tuberculin sensitivity in guinea pigs, that adrenocorticotrophic hormone and cortisone exert their beneficial actions by facilitating conversion of ascorbic acid into dehydroascorbic acid.

Therefore, when relatively pure preparations of dehydroascorbic acid became available, it was decided to study the degree of its reduction to ascorbic acid when administered orally or by injection to guinea pigs and to human subjects and whether adrenocorticotrophic hormone (ACTH) or cortisone would inhibit this reduction. The possible effects of adrenocorticotrophic hormone and cortisone favouring the oxidation of injected ascorbic acid were also studied.

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