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THE STABILITY OF ENDOGENOUS CORTICOTROPHIN IN RAT BLOOD *IN VITRO*

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Endogenous corticotrophin is stable in blood *in vitro* for periods of up to 1 hr. at 4° and 22°, and in plasma for at least 24 hr. at −15°. Estimates of plasma corticotrophin concentration provide less consistent results than assays done on whole blood. The optimal conditions for storing blood prior to the determination of its corticotrophin concentration are discussed.

There is some confusion in the literature concerning the stability of corticotrophin in blood *in vitro*. Several studies on commercial samples of corticotrophin added to blood have had conflicting results. However, very little information has been published on the stability in blood *in vitro* of the *endogenously* secreted hormone. The present work was done because such information is essential to workers concerned with the estimation of circulating corticotrophin. Experiments are described in which blood samples removed from adrenalectomized and stressed adrenalectomized rats were kept under various conditions and then assayed for corticotrophin content.

METHODS

Albino Wistar rats weighing 120 to 160 g. were used in all the experiments. They were fed on a diet of cubes (diet 41, Lane-Petter and Dyer, 1952) and water, and were kept in a room at a constant temperature of 22° for at least one week before use. Approximately 200 female rats were used to provide the blood samples and 270 males for the corticotrophin assays.

Female rats were adrenalectomized bilaterally under ether anaesthesia and were maintained subsequently on the normal diet with 0.9% sodium chloride solution in place of drinking water. Blood was obtained 30 days after adrenalectomy from conscious rats or from animals which had been anaesthetized with ether 2.5 min. previously. The animals were decapitated and blood was collected from their trunks into tubes containing heparin. When plasma samples were used, the plasma was separated after centrifugation of the blood at 3,000 rev./min. for 15 min. Approximately one half of each pooled blood or plasma sample (control) was immediately injected intravenously into groups of at least 6 male rats which had been pretreated with hydrocortisone (Hodges and Vernikos, 1958; Cox, Hodges, and Vernikos, 1958). The remaining half (test) was kept in pyrex glass containers at 4° or 22° for times varying from 40 to 320 min. (whole blood), or at −15° for 24 hr. (plasma), before being similarly injected into hydrocortisone treated rats. A third group of assay rats received injections of normal saline only. All the injections were administered in doses of 3 ml./100 g. body weight. The injected animals were killed 1 hr. later and their adrenal ascorbic acid contents were determined by the method of Roe and Kuether (1943). The mean adrenal ascorbic acid in each group of rats which had received the blood or plasma samples was subtracted from the mean of the corresponding saline injected group. The adrenal ascorbic acid depletion produced by each sample was taken as a measure of its corticotrophin content.

RESULTS

The mean adrenal ascorbic acid depletions produced by blood and plasma samples from adrenalectomized and stressed (ether 2.5 min.) adrenalectomized rats before and after storage for various times at −15°, 4°, and 22° are shown in Table I. The fall in adrenal ascorbic acid induced by the control samples of blood, obtained from adrenalectomized rats and injected immediately after collection, varied between 70 ± 11 and 90 ± 12 mg./100 g. adrenal tissue (mean ± S.E.). None of these results differed significantly ($P<0.05$) from one another. Blood samples from adrenalectomized rats kept at 22° for 40 and 80 min. produced adrenal ascorbic acid depletions which were not significantly ($P<0.05$) less than those caused by the control samples. However, similar blood samples stored at the same temperature for 160 min. possessed only about one

TABLE I

STABILITY OF ENDOGENOUS CORTICOTROPHIN

Adrenal ascorbic acid depletions in hydrocortisone treated rats 1 hr. after intravenous injections (3 ml./100 g. body weight) of blood and plasma, before (controls) and after (tests) storage at various times and temperatures. Means±S.E. No. of rats in parentheses. The ascorbic acid level in the saline-injected controls was 505±11 mg./100 g. and in uninjected hydrocortisone treated rats 508±11 mg./100 g. adrenal tissue.

Material Assayed	Storage Temp. (°C.)	Storage Time (min.)	Mean Depletion (mg./100 g. Tissue±S.E.)	
			Control	Test
Blood from adrenalectomized rats	22	40	87± 6 (16)	81± 7 (17)
,, ,,	22	80	89± 7 (13)	91± 5 (16)
,, ,,	22	160	71± 8 (11)	29± 9 (9)
,, ,,	22	320	90±12 (6)	−16±20 (6)
,, ,,	4	160	70±11 (6)	52±11 (6)
,, ,,	4	320	90±12 (6)	20± 5 (6)
Blood from stressed adrenalectomized rats ..	22	40	139± 7 (6)	124± 9 (6)
,, ,,	22	80	139± 5 (24)	132± 8 (11)
,, ,,	22	160	139± 7 (6)	36±11 (6)
Plasma from stressed adrenalectomized rats	−15	24 hr.	98±11 (6)	121±13 (6)

TABLE II

COMPARISON OF PLASMA AND WHOLE BLOOD

Adrenal ascorbic acid depletions in hydrocortisone treated rats 1 hr. after intravenous injections (3 ml./100 g. body weight) of blood, plasma, saline washings, and suspensions of cell sediments from same samples. Means±S.E. No. of rats in parentheses.

Expt. No.	Material Assayed	Mean Depletion (mg./100 g. Tissue ±S.E.)
1	Whole blood from normal intact rats ..	−11± 7 (6)
	Plasma from same sample	−5±12 (6)
	Cell sediment from same sample resuspended in normal saline	16±14 (6)
2	Whole blood from stressed adrenalectomized rats	129±14 (6)
	Plasma from same sample	158± 9 (5)
	Saline washings of cell sediment ..	99± 8 (6)
	Washed sediment resuspended in normal saline	91± 9 (6)
3	Whole blood from stressed adrenalectomized rats	134±10 (6)
	Plasma from same sample	98±11 (6)
4	Whole blood from stressed adrenalectomized rats	109± 7 (6)
	Plasma from same sample	106±12 (6)
	Cell sediment from same sample resuspended in normal saline	57±10 (6)

half of the adrenal ascorbic acid depleting activity of the controls, and samples kept for 320 min. produced no fall in adrenal ascorbic acid. Storage of blood at 4° delayed slightly the rate of inactivation. Samples of blood kept at this temperature for 160 and 320 min. possessed about three-quarters and a quarter of the activity of the controls respectively. Similar results were obtained using blood from stressed adrenalectomized rats, but the adrenal ascorbic acid depleting activity of the control samples was greater. About 50% inactivation occurred at 22° after storage for 80 to 160 min. Plasma stored at −15° retained its entire activity for 24 hr.

Table II shows the results of experiments in which the adrenal ascorbic acid depletions produced by whole blood were compared with those due to plasma and washings and suspensions of the cell sediments from the same blood samples. Volumes of normal saline equal to that of the separated plasma were used to wash or suspend the sediments.

Blood, plasma, and cell suspensions from normal animals possessed no detectable activity. Whole blood from stressed adrenalectomized rats caused a pronounced fall in adrenal ascorbic acid concentration and the results of several experiments did not differ significantly ($P<0.05$). However, the results obtained using plasma were more variable. Considerable activity was left in the cell fraction, and saline washings and suspensions of the cell sediments produced marked adrenal ascorbic acid depletion. No explanation can be suggested for the anomalous result of experiment 2 (Table II), where the total activity in the plasma, washings, and suspensions of the cell sediments appeared to be greater than that in the whole blood. However, this is the only instance in our experience where the plasma corticotrophin level was markedly higher than that in blood.

DISCUSSION

There are relatively few stability studies on corticotrophin. Some investigators have examined the stability *in vitro* of exogenous corticotrophin added to blood. Reiss, Badrick, Halkerston, and Plaice (1951) reported the extreme instability of the hormone in such conditions, and Pincus (1951) observed the presence in blood of an enzyme capable of destroying corticotrophin. On the other hand, Richards and Sayers (1951), Pincus, Hechter, and Hopkins (1952) and Geschwind and Li (1952) found that incubation of corticotrophin with plasma did not result in any appreciable loss of activity. These discrepancies may possibly be explained on the basis that different preparations of the hormone, varying considerably in source and purity, were used in the investigations. Certainly the results yielded information of little value to workers concerned with the estimation of endogenously secreted corticotrophin in blood.

Direct estimates of circulating corticotrophin are now being used more frequently by laboratory workers investigating the physiology of the

pituitary-adrenal system. Furthermore, the development of simpler and more sensitive assay techniques makes it probable that routine methods for the estimation of blood corticotrophin will ultimately be available to clinical endocrinologists. It appeared, therefore, that the stability in blood of endogenously secreted corticotrophin should be systematically studied to obtain more information on the optimal conditions for the collection and storage of blood samples before the determination of their corticotrophin content.

Adrenalectomized and stressed adrenalectomized rats were used in the present study to provide the blood samples because their circulating levels of corticotrophin are sufficiently high to be estimated easily by the method described by Cox *et al.* (1958). The concentration of corticotrophin in the blood of rats three weeks after adrenalectomy is approximately 10 mU./100 ml. of blood (Cox *et al.*, 1958), and this high circulating level of the hormone is raised even further 2.5 min. after anaesthesia with ether (Hodges and Vernikos, 1959).

From a consideration of Table I it appears that there is no appreciable loss of adrenocorticotrophic activity when whole blood is stored at temperatures between 4° and 22° for periods of up to about 1 hr. or when plasma is kept at −15° for 24 hours. These results are in agreement with the findings of Paris (1954), who showed that plasma withdrawn from adrenalectomized rats possessed considerable adrenocorticotrophic activity 30 min. later. Montanari, Martinelli, Rossi, and Moruzzi (1951) reported that normal human plasma contained 160 mU. corticotrophin/100 ml. and that 49% of this activity was lost 4 hr., and 100% 12 hr., after storage at 18°. The validity of their results is placed in doubt by the fact that few other workers have been able even to detect corticotrophin in normal human plasma.

It was impracticable to study the stability of corticotrophin in whole blood stored at −15°, since changes, such as haemolysis, which occur make it too toxic to be tolerated by the assay animals. In our experience rat serum is also toxic when injected intravenously into rats in volumes greater than 0.5 ml./100 g. of body weight although Clayton, Hammant, and Armitage (1957) found that guinea-pig serum "led to anaphylactic reactions with death" only in doses greater than 2.0 ml./100 g.

Since only plasma can be stored in the conditions necessary for maximal stability of corticotrophin, it would appear desirable to perform corticotrophin assays on plasma rather than on whole blood. Furthermore, Barrett (1956) observed that the separation of plasma had the added advantage of concentrating corticotrophin added to whole blood, presumably since the added hormone was distributed only throughout the plasma. However, in our experience estimates of plasma corticotrophin are more variable than determinations of the hormone in whole blood. Our results also provide very little evidence that the concentration of corticotrophin is higher in the plasma fraction. It appears that much of the hormone remains associated with the cellular fraction, probably adsorbed on to the surface of the cells. This is consistent with the observation that considerable activity can be demonstrated in saline washings or suspensions of the cells.

These studies do not suggest how corticotrophin is inactivated by blood. The destruction of crude preparations of corticotrophin added to blood has been attributed to enzymes which they contain (Pincus *et al.*, 1952), and probably endogenous corticotrophin is inactivated similarly. The disappearance of corticotrophin from the circulation *in vivo*, however, occurs much more rapidly, and most estimates of the biological half-life of the hormone in blood vary between 1 and 5 min. (Greenspan, Li, and Evans, 1950; Gemzell, Van Dyke, Tobias, and Evans, 1951; Sydnor and Sayers, 1953; Sydnor, 1955).

Astwood (1955) considered that, since the increased content of corticotrophin in the blood following stress and adrenalectomy is thought to disappear from the blood at such a rapid rate, "rigorous precautions are essential to prevent this if assays are to be made." The results of the present series of experiments indicate that elaborate precautions are not necessary. Assays of circulating corticotrophin should be made on whole blood rather than on plasma. Excess heparin should be added to the blood samples to prevent clotting and the release of toxic substances, the samples should be kept cool, and the assays should be made within an hour of collection. If these very simple precautions are observed, estimates of circulating corticotrophin may be made with no reason for doubting their validity.

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