ethyl and propyl esters (compounds *40*, *41*) come next. However, in general they have lower fungistatic activity than the alkyl naphthols.

Comparing the activities against all the fungi included in the tests (Table III), it is observed that while compounds *8* and *14*, 3-butyl- and 1-methyl-6-propyl-β-naphthol, are fungistatic at 20 mcg./ml., all others are fungistatic only at 50 mcg./ml.

REFERENCES

(1) Baichwal, R. S., and Khorana, M. L., *J. Sci. Ind. Res. (India)*, **11B**, 169(1952).
(2) *Ibid.*, **12B**, 43(1953).
(3) Baichwal, R. S., Baichwal, M. R., and Khorana, M. L., *Indian J. Pharm.*, **16**, 93(1954).
(4) Baichwal, R. S., Baichwal, M. R., and Khorana, M. L., *J. Am. Pharm. Assoc., Sci. Ed.*, **46**, 603(1957).
(5) *Ibid.*, **47**, 537(1958).
(6) Baichwal, R. S., Khorana, M. L., and Pishawikar, A. D., *Indian J. Pharm.*, **18**, 224(1956).
(7) Khorana, M. L., and Pishawikar, A. D., *ibid.*, **23**, 294(1961).
(8) *Ibid.*, **23**, 297(1961).
(9) Khorana, M. L., and Pandit, S. Y., *J. Indian Chem. Soc.*, **40**, 789(1963).
(10) Khorana, M. L., and Pandit, S. Y., *Indian J. Chem.*, **1**, 489(1963).
(11) Khorana, M. L., Pandit, S. Y., and Pishawikar, A. D., *ibid.*, **2**, 410(1964).
(12) McKee, C. M., Rake, C. P., and Menzel, A. E., *J. Immunol.*, **48**, 259(1944).
(13) Burlingame, E. M., and Reddish, G. F., *J. Lab. Clin. Med.*, **24**, 771(1939).
(14) Schamberg, J. F., and Kolmer, J. A., *Arch. Dermatol. Syphilol.*, **6**, 746(1922).

Effects of Adrenochrome Semicarbazone on Blood Loss in the Mouse

By JACK H. KEITH, WILLIAM W. KEITH, and MARY H. NELSON

A method for determining the blood loss and bleeding time of a standardized wound is described, and it is applied in examining the activities of a clinically-used hemostatic agent—adrenochrome semicarbazone. It is shown that adrenochrome semicarbazone has an effect in decreasing blood loss at doses as low as 0.1 mg./Kg., and is effective in the presence of anticoagulants. A mechanism of action is suggested.

THE DIFFICULTY in showing a decrease in blood loss, clinically or experimentally, is apparent when one attempts to produce a standard wound. Variations of up to 1000% may occur on the same animal with the same size wound.

Roskam (1) measured blood loss from rabbit ears, achieving reproducible results, but the authors found that his method gave insufficient information. The major deficiency in his procedure was the measurement of bleeding times rather than blood loss. Blood loss was assumed to parallel bleeding time, and the authors have found that this is not always true.

After many attempts at determining blood loss, with and without adrenochrome semicarbazone, it was found that blood loss could be most accurately and reproducibly measured in mice. The clipping of 1.5 cm. from the tips of the tails of animals of the same size and sex gives an easily measurable method of determining blood loss.

The mouse tail was used because the vessels are large enough to produce a bleeding condition that could be classified as traumatic hemorrhage. The major vessels in the mouse tail vary from 0.05 mm. to 0.25 mm. in diameter. During the peak period of hemorrhage, the first 30 sec., the blood loss may be as great as 0.2 ml., which would represent 10% of total blood volume.

This paper concerns the effect of adrenochrome semicarbazone upon initial blood loss from the clipped tail of the mouse, and an indication of its mechanism of action. We emphasize the term "initial blood loss," since anticoagulant-treated animals stop bleeding only for a short period, and then begin again.

METHOD

Determination of Blood Loss and Bleeding Time—Male mice[1] weighing 18–22 Gm. were used. The tail was severed 1.5 cm. from the tip with a frequently changed razor blade to ensure keenness. The animals were placed in a holder at least 5 min. before cutting. This holder consisted of a plastic vial 1 in. × 2.5 in. (Fig. 1) with holes for ventilation. In some of the experiments the tails were bled into a beaker containing 100 ml. of deionized water at 37°; 3–5 mice were bled into the same 100 ml. of water containing disodium ethylendiaminetetraacetate, 2 mg./100 ml., as an anticoagulant. The tail was observed until bleeding stopped and was then removed immediately from the water. The

Received December 5, 1966, from the Research Division, S. E. Massengill Co., Bristol, TN 37621
Accepted for publication May 19, 1967.

[1] ICR strain random breed, obtained from Dublin Animal Farm, Dublin, Va.

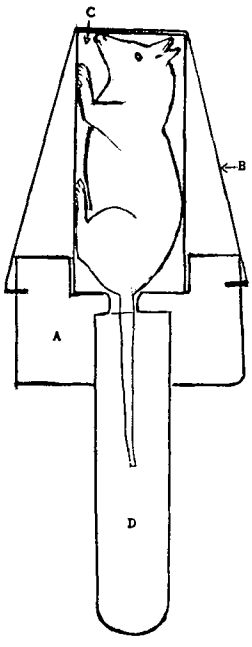


Fig. 1—Holder and tube arrangement for bleeding-time determinations. Key: A, Plexiglas block 5.5 cm.² and 3 cm. thick. When used with a 100-ml. beaker the block is 1.5 cm. thick; B, wire holder, 19 mm. for test tube and 29 mm. for mouse holder; C, plastic vial, 64 mm. × 28 mm.; D, 25-ml. test tube.

dose response experiment varied from this procedure in that the tail was left in the water for 3 min. After bleeding, 1 ml. of concentrated hydrochloric acid was added to the beaker to change the hemoglobin to acid hematin for quantitative measurement. The blood loss was determined from a curve made from a pooled sample of known quantities of blood which was read at 540 mμ on a Bausch & Lomb spectrophotometer (Spectronic 20).

In some of these tests a 25-ml. tube was used in place of the 100-ml. beaker, and only one mouse was bled into each 25 ml. of water.

The mean values in all tests were compared, using the Fisher *t* test for significance (2).

Dose Response—The drug was administered subcutaneously at 0.1, 1, and 5 mg./Kg. Fifteen minutes were allowed for drug action. The mouse tail remained in the water for 3 min.

Adrenochrome Semicarbazone and Heparin Administration—The mice were given 6 mg./Kg. sodium heparin intraperitoneally (119 units/mg.) and 30 min. was allowed for drug action. Adrenochrome semicarbazone was used at a 5 mg./Kg. dosage subcutaneously, allowing 30 min. for drug action.

Adrenochrome Semicarbazone and Bishydroxycoumarin Administration—The effects of bishydroxycoumarin in mice were investigated by administering the drug orally or intraperitoneally daily for 2 to 8 days. One group (A) was given the bishydroxycoumarin in the diet at a level of 0.015% for 8 days. In two of the groups (B), bishydroxycoumarin was given intraperitoneally, 20 mg./Kg. at 0 hr. and 10 mg./Kg. at 24 hr. The mice were bled 24 hr. after the second dose. In three other groups (C), a 10-mg. dose intraperitoneally was given at 0, 24, and 48 hr.; the mice were bled at 72 hr. Adrenochrome semicarbazone, 0.5 mg./ml., was added to the drinking water and 3 mg./Kg. was injected subcutaneously 30 min. before bleeding.

Each group—controls, bishydroxycoumarin, and bishydroxycoumarin–adrenochrome semicarbazone—contained 21 mice. Three mice were bled into the same 100 ml. of water.

Adenosine Diphosphate and Adenosine Administration—These drugs were given at 1 and 30 mg./Kg. intraperitoneally in 2% gelatin solution. Thirty minutes was allowed for drug action. Forty animals were used at each dosage level and compared with 40 controls receiving an equal volume of 2% gelatin solution.

Bishydroxycoumarin Toxicity—One hundred male mice, 22–25 Gm., were given their normal diet *ad libitum* with bishydroxycoumarin 80 mg./100 Gm. of food. Another group of 100 mice received bishydroxycoumarin, 80 mg., and adrenochrome semicarbazone, 60 mg./100 Gm. of food. The number of deaths were recorded daily for 8 days. The drug was removed from the food on the 6th day. These animals were caged in groups of 10. (See Table IV.)

In another test the mice were caged individually and fed the regular diet plus bishydroxycoumarin, 35 mg./100 Gm. of food. One group was given adrenochrome semicarbazone at a level of 20 mg./100 Gm. of food. The drugs were administered for 12 days. Each of the two groups contained 75 animals. (See Table V.)

The daily accumulative deaths were graphed using the Litchfield–Wilcoxon (3) method to analyze the LD effects for possible difference. The day of death rather than the drug consumption was used as the abscissa. The LD_{50} is based on the number of days after beginning drug administration.

Heparin Toxicity—Male mice, 22–25 Gm., were injected with adrenochrome semicarbazone subcutaneously, 5 mg./Kg. in a volume of 0.5 ml./100 Gm. body weight. The adrenochrome semicarbazone was given 5–10 min. before intravenous administration of sodium heparin. The tails were cut with a razor blade 2.5–3 cm. from the base immediately after the injection of the heparin. Those animals given only heparin were given a volume of physiological saline, 0.5 ml./100 Gm., subcutaneously. The sodium heparin was dissolved in 0.85% sodium chloride solution and administered at a volume of 1 ml./100 Gm. of body weight. The dosage levels of heparin were 500, 1000, 1500, 2000, and 3000 units/Kg. The animals, in groups of 10, were placed in plastic boxes 5 × 6 × 10 in. with shavings on the floor. The results were graphed, and the Litchfield-Wilcoxon (3) method of comparison was used to determine significance of difference in the two treatments.

RESULTS AND DISCUSSION

The end point in these experiments is easily determined, but accounting for the wide variations in blood loss is a complex, unsolved problem. The day-by-day differences in groups often reaches a multiple of three in treated and untreated alike.

Dose Response—Table I shows a dose response obtained with adrenochrome semicarbazone as seen in the mouse tail experiment. To make this test more objective, the blood from each mouse was collected for a period of 3 min. at which time the

TABLE I—EFFECT OF ADRENOCHROME SEMICARBAZONE ON BLOOD LOSS IN NORMAL MICE[a]—A DOSE RESPONSE

	Controls	Adrenochrome Semicarbazone 0.1 mg./Kg.	1 mg./Kg.	5 mg./Kg.
Blood Loss, ml.	.195	.107	.085	.087
S.D.	.130	.084	.041	.051
T Value		5.5[b]	4.5[c]	
Significance		.01	.01	

[a] Number of animals used: controls, 74; adrenochrome semicarbazone 0.1 mg./Kg., 101; 1 mg./Kg., 33; 5 mg./Kg., 39. [b] As compared to controls. [c] As compared to 0.1 mg./Kg.

TABLE II—EFFECTS OF ADRENOCHROME SEMICARBAZONE IN HEPARIN-TREATED MICE[a]

	Controls	Heparin, 6 mg./Kg.	Heparin, 6 mg./Kg., and Adrenochrome Semicarbazone, 5 mg./Kg.
Blood loss, ml.	0.142	0.114	0.086
T value		1.4[b]	2.1[c]
Significance		0.2	0.05
Bleeding time, sec.	100	138	114
T value		2.4[b]	2.5[c]
Significance		0.01	0.02

[a] Each group contained 81 animals which were bled in groups of three. [b] As compared to controls. [c] As compared to heparin alone.

TABLE III—EFFECTS OF ADRENOCHROME SEMICARBAZONE ON LD EFFECT OF HEPARIN IN TRAUMATIZED MICE

Heparin, Units/Kg. i.v.	Heparin[a]	Heparin and Adrenochrome Semicarbazone, 5 mg./Kg.[a]
500	1/10	1/10
1000	2/10	4/10
1500	12/20	13/20
2000	15/20	17/20
3000	9/10	10/10
LD_{50}	1150 Units	1400 Units
	Potency ratio $= \frac{1400}{1150} = 1.2$	
	Factor of potency ratio = 1.22	

[a] Number dead/number used.

tail was removed from the water. The 3-min. time period is considerably longer than the normal bleeding time. The dosage of 0.1 mg./Kg. is in the same range as that used clinically.

With a drop in blood loss of 45% at 0.1 mg./Kg., it might be concluded that a lower dose would have effected a dose response. Constantinescu (4) *et al.* have found activity at much lower doses in rats. They compared blood loss from the tail in anesthetized rats, 6 groups of 40 each, and found a 64% reduction in blood loss at 5 mcg./Kg. adrenochrome semicarbazone.

Heparin—Table II shows that adrenochrome semicarbazone decreases blood loss in heparin-treated mice. Bleeding time is significantly increased by heparin, but the initial blood loss is less than that of the controls. The blood pressure is lowered by doses of heparin larger than those used in this experiment, which in itself will reduce initial blood flow. The authors have observed in previous experiments that a dose of 25 mg./Kg. of heparin will cause initial blood loss to fall to a very low level. Care was exercised in all the experiments to rule out an effect due to a drop in blood pressure. Mice given 6 mg./Kg. doses of heparin occasionally bleed to death after the tail is severed. The blood stops flowing, but a clot does not form and the bleeding resumes. If the tail is observed for long periods, there are numerous periods of spontaneous bleeding. Although there is a decrease in the initial blood loss, the LD_{50} of heparin in mice is not changed when adrenochrome semicarbazone is administered. In addition to the anticoagulant effect of heparin, which exceeds 1 hr. in these experiments, Zucker (5) and Solandt and Best (6) have shown that the activity of platelets is greatly decreased.

To determine an LD_{50} with heparin, a standard wound is produced by severing the tail 2.5–3 cm. from the base. Those animals that succumb do so in a period of 2–12 hr. The wound greatly reduces the amount of heparin necessary for a lethal dose. The authors had previously noted that doses of heparin, 4000–12,000 units/Kg., do not produce death, but often a semishock state is produced. In many mice there is a massive fluid exudate in the peritoneal cavity. Because of this fluid loss, the dosage was kept below 4000 units/Kg. and a trauma severe enough to cause death was used.

The LD_{50} for the heparin–adrenochrome combination was 1150 units/Kg., and 1400 units/Kg. for the heparin only group. (See Table III.) The difference of 350 units/Kg. in toxicity was not significant at the 95% confidence limits.

Bishydroxycoumarin—When bleeding time and blood loss is examined in groups of mice given bishydroxycoumarin and adrenochrome semicarbazone (Table VI), there is a significant decrease in blood loss in these animals as compared with bishydroxycoumarin-treated animals, with the bleeding time returned to normal. An exception was noted in group A: some of these animals had died and the remainder were in severely hemorrhagic condition. Hemorrhagic areas were present around the shoulders and testicles, and in the peritoneal cavity. The bleeding time increased significantly with bishydroxycoumarin, but blood loss did not reflect this increase. The results seen here with bishydroxycoumarin agree with those seen by Chen and Tsai (7). They state that in their animals (frogs and rabbits) given bishydroxycoumarin the initial bleeding was stopped by vasoconstriction, but bleeding started again when the constricted vessels relaxed. Without intervention death often resulted.

As indicated in Tables II and III, the failure of bishydroxycoumarin and heparin to increase the initial blood loss is a convincing demonstration of the importance of factors other than clotting mechanisms in the initial retardation of blood flow.

The ability of adrenochrome semicarbazone to reduce initial blood loss in heparin- or bishydroxycoumarin-treated animals clearly indicates activity in the absence of effective clotting components.

TABLE IV—EFFECTS OF ADRENOCHROME SEMICARBAZONE ON BISHYDROXYCOUMARIN LD_{50} EFFECT

Days[a]	Bishydroxycoumarin, 80 mg./100 Gm. Food No. Dying	A.P.D.[b]	Bishydroxycoumarin, 80 mg., and Adrenochrome Semicarbazone, 20 mg./100 Gm. Food No. Dying	A.P.D.[b]
3	5	5	7	7
4	9	14	9	16
5	8	22	14	30
6	20	42	9	39
7	10	52	12	51
8	2	54	4	55
Deaths	54		55	
Time of LD_{50}, days	7.6		7.25	
Time ratio	7.6/7.25 = 1.047			
Factor of time ratio	1.28			

[a] Number of days of drug administration. [b] Accumulative per cent deaths.

TABLE V—EFFECTS OF ADRENOCHROME SEMICARBAZONE ON BISHYDROXYCOUMARIN LD_{50} EFFECT

Days[a]	Bishydroxycoumarin 35 mg./100 Gm. Food No. Dying	A.P.D.[b]	Bishydroxycoumarin, 35 mg., and Adrenochrome Semicarbazone, 20 mg./100 Gm. Food No. Dying	A.P.D.[b]
4	3	4	6	8
5	3	8	8	18
6	24	40	26	53.5
7	1	41	1	54.5
8	2	44	4	60
9	3	59	3	64
10	17	71	3	68
11	1	72	9	79
12	1	74	1	80
Deaths	55		60	
Time of LD_{50}, days	6.7		6	
Time ratio	6.7/6 = 1.17			
Factor of time ratio	1.25			

[a] Number of days of drug administration. [b] Accumulative per cent deaths.

The toxicity of bishydroxycoumarin requires several days to be manifest. (See Tables IV and V.) Thus the drug was administered in the diet. Food consumption was normal the first 3 days with a sharp drop after that. There was no difference in quantity of food consumed by the 2 groups. The animals receiving bishydroxycoumarin, 35 mg./100 Gm. of food, were not less affected than those given 80 mg./100 Gm. of food. This was not expected, since the animals at 35 mg. were caged individually. The authors anticipated that individual contact would intensify hemorrhage, causing a more rapid death. It may be that grouping together helps the animals to maintain normal temperature. The time required for bishydroxycoumarin to produce death in 50% of the mice was not reduced by adrenochrome semicarbazone.

Since adrenochrome semicarbazone fails to reduce mortality in heparin- or bishydroxycoumarin-treated animals, the activity would have to be other than an antiheparin or increased coagulation effect. The mechanism left to consider would be an effect on increasing the efficiency of the platelet plug formation or potentiation of the local vasoconstrictor effect.

Adenosine and Adenosine Diphosphate—Stewart and Brooks (8) presented information suggesting that adrenochrome semicarbazone produced a hemostatic effect in irradiated rats by increasing the activity of platelets. In the authors' test, the effects appeared to be separate from the platelet plug effect. This is not to infer that adrenochrome semicarbazone does or does not have an effect on or in conjunction with platelets, but that adrenochrome semicarbazone will promote hemostasis in the absence of the platelet plug.

Johnson (9) *et al.* have recently observed the mechanism of hemostasis in vessels of 50–160 μ by sectioning the hemostatic plug. Their plug was composed of red cells enmeshed in fibrin. Platelet aggregation did not occur until after this plug formed.

Other evidence which helps us rule out more efficient plug formation as the basic method of adrenochrome semicarbazone activity was obtained with adenosine and adenosine diphosphate. Adenosine diphosphate, a physiological substance known to produce *in vivo* clumping of platelets (10–12) and the subsequent platelet plug, was used to test the effect of increased platelet activity. With adenosine diphosphate, the initial blood loss was not decreased. Adenosine is a competitive inhibitor (12) of adenosine diphosphate and thereby blocks the formation of the platelet plug. Adenosine did not increase the initial blood loss. These results strongly suggest that the hemostatic effect of adrenochrome semicarbazone in the experiments was not in the more rapid building of the platelet plug but on the local vascular contraction at the area of the trauma.

TABLE VI—EFFECTS OF ADRENOCHROME SEMICARBAZONE ON BLEEDING TIME AND BLOOD LOSS IN BISHYDROXYCOUMARIN-TREATED MICE

Group	Controls B.T., sec.	Controls B.L., ml.	Bishydroxycoumarin B.T., sec.	Bishydroxycoumarin B.L., ml.	Bishydroxycoumarin and Adrenochrome Semicarbazone B.T., sec.	Bishydroxycoumarin and Adrenochrome Semicarbazone B.L., ml.
A[a]	99	.392	128	.120	109	.119
B[b]	97	.200	130	.342	81	.024
B	118	.159	138	.157	105	.054
C[c]	101	.255	158	.319	123	.140
C	97	.319	138	.391	103	.239
C	128	.335	93	.178	108	.107
Av.	106	.276	130	.251	104	.114
T Value		3.8		2.5		
Significance		.01		.05		

Received bishydroxycoumarin for 8 days. [b] Bishydroxycoumarin 20 mg./Kg. at 0 hr., 10 mg./Kg. at 24 hr.; bled at 48 hr. [c] 10 mg. at 0, 24, 48 hr.; bled at 72 hr.

SUMMARY

Adrenochrome semicarbazone has been shown to decrease blood loss in normal mice whose tails have been clipped. The decrease in blood loss does not correlate with bleeding time. The bleeding times increased with anticoagulants but initial blood loss did not change significantly.

Heparin and bishydroxycoumarin have been used to deplete or block the blood stream of necessary clotting components and in these animals adrenochrome semicarbazone can be shown to decrease the blood loss when measured to cessation of the initial blood flow. Adrenochrome semicarbazone will not increase rate of survival in animals lacking a necessary amount of clotting components.

The failure of adenosine and adenosine diphosphate, heparin, and bishydroxycoumarin to influence initial blood loss indicates that in the tail vessel of the mouse the primary hemostatic consideration is that of local vasoconstriction. Adrenochrome semicarbazone appears to augment this constrictor effect.

REFERENCES

(1) Roskam, J., "L'Hemostase Spontanee," Masson & Cie Editeor, Paris, France, pp. 63–64, 81–84.
(2) Fisher, R. A., "Statistical Methods for Research Work," Oliver & Boyd, London, England, 1950, p. 122.
(3) Litchfield, J. T., Jr., and Wilcoxon, F., *J. Pharmacol. Exptl. Therap.*, **96**, 99(1949).
(4) Constantinescu, M., Stoian, F., Papa, V., and Copae, L., *Pharmazie*, **16**, 458(1961).
(5) Zucker, M. B., *Am. J. Physiol.*, **148**, 275(1947).
(6) Solandt, D., and Best, C., *Lancet*, **1**, 1042(1942).
(7) Chen, T. L., and Tsai, C., *J. Physiol.*, **107**, 280 (1948).
(8) Brooks, A. M., and Stewart, G. J., *Fed. Proc. Abstr.*, **25**, 2898(1966).
(9) Johnson, S. A., Fredell, L. M., Shepard, J. A., Tebo, T. H., Pedrson, H. J., and VanHorn, D. L., Fourteenth Annual Symposium on Blood, Wayne State University of Medicine, January 1966, p. 6.
(10) Spaet, T. H., *ibid.*, p. 16.
(11) Bambel, D. E., *ibid.*, p. 16.
(12) Haslam, R. J., *ibid.*, p. 10.

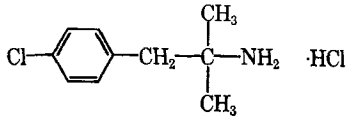
Drug Standards

Qualitative and Quantitative Tests for Chlorphentermine Hydrochloride

By EDWARD F. SALIM*, J. RITNER WEAVER†, and LESTER CHAFETZ†

Provisional, unofficial monographs are developed by the Drug Standards Laboratory, in cooperation with the manufacturers of the drug concerned, for publication in the *Journal of Pharmaceutical Sciences*. The ready availability of this information affords discriminating medical and pharmaceutical practitioners with an added basis for confidence in the quality of new drug products generally, and of those covered by the monographs particularly. Such monographs will appear on drugs representing new chemical entities for which suitable identity tests and assay procedures are not available in the published literature. The purity and assay limits reported for the drugs and their dosage forms are based on observations made on samples representative of commercial production and are considered to be reasonable within expected analytical and manufacturing variation.

p-CHLORO-α,α-DIMETHYLPHENETHYLAMINE HYDROCHLORIDE; $C_{10}H_{14}ClN.HCl$; mol. wt. 220.14. The structural formula of chlorphentermine hydrochloride may be represented as:



Physical Properties—Chlorphentermine hydrochloride occurs as an odorless, white to off-white powder with a bitter taste, m.p. 232–235° (U.S.P. class I). It is freely soluble in water and in alcohol, sparingly soluble in chloroform, and practically insoluble in ether. The pH of a solution of chlorphentermine hydrochloride in carbon dioxide-free water (1 in 100) is between 5.0 and 6.0.

Received August 16, 1966, from the * Drug Standards Laboratory, AMERICAN PHARMACEUTICAL ASSOCIATION FOUNDATION, Washington, DC 20037
Accepted for publication May 10, 1967.
† Warner-Chilcott Laboratories, Morris Plains, NJ 07950.
Warner-Chilcott Laboratories has cooperated by furnishing samples and data to aid in the development and preparation of this monograph.
The Drug Standards Laboratory gratefully acknowledges the assistance of Miss Carolyn Damon and Miss Hannah Klein.