THE INACTIVATION OF PENICILLINS F, G, K, AND X BY HUMAN AND RABBIT SERUM

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It has been repeatedly stated (1–5) that the bactericidal activity of penicillin is not demonstrably affected by serum, blood, living tissue cells, or their autolytic products. In 1944, however, Bigger (6) reported that when commercial sodium penicillin was incubated at 37°C. in 80 to 90 per cent human serum, its bactericidal activity (determined with *Staphylococcus pyogenes* in a plate assay) slowly fell off at the rate of approximately 5 per cent per hour. Similarly, Holmes and Lockwood (7) reported that human, horse, and rabbit sera interfered with the staphylococcidal action of penicillin. Most workers have, however, commented on the fact that serum or blood does not inhibit the action of penicillin, and in one study (3) its bactericidal action was said to be at times actually enhanced.

These discrepant reports, and the paradoxical finding that penicillin K is therapeutically almost inactive (8–11) despite a high measure of bactericidal activity *in vitro* (11, 12) led us to reinvestigate the susceptibility of penicillin to inactivation by blood, but using crystalline penicillins F, G, K, and X instead of the mixture of penicillins present in the commercial products hitherto available. As will be shown in the present paper, all four penicillin species are inactivated by human or rabbit serum, plasma, or whole blood. The inactivation of F, G, and X is relatively slow, and from 70 to 100 per cent of these three penicillins is excreted in the urine in active form after their administration in therapeutic dosage. Penicillin K, however, is rapidly inactivated by serum (or plasma) *in vitro*. Probably in consequence of a similar inactivation *in vivo*, it disappears from the blood far more rapidly than either F, G, or X, its urinary excretion usually falls off abruptly after the first 30 to 60 minutes, and a total of only 20 to 40 per cent is excreted in the urine in active form. There is reason to believe that the inactivation of penicillin K by blood or serum differs qualitatively as well as quantitatively from that of F, G, and X.

The data here presented provide a simple explanation for the paradoxical inactivity of penicillin K *in vivo*. The relatively minor but significant differences in the susceptibility of penicillins F, G, and X to inactivation by serum,

and the implications of these findings with respect to their therapeutic activity, are discussed in the text.

Methods and Materials

Penicillin Assay in Urine and Blood.—A serial dilution technic resembling the methods of Rammelkamp (13) and Kirby and Rantz (14) was used for the assay of peniciillins F, G, K, and X in blood and urine. *Streptococcus pyogenes* (strain C-203) was used as the test organism, and the presence or absence of hemolysis as the end-point (*cf.* reference 12). In the present experiments, five dilutions were interpolated for each twofold difference in concentration (*i.e.*, the unknown specimen in appropriate dilution was distributed in the amounts of 0.8, 0.72, 0.6, 0.48, 0.4, 0.36, 0.3, 0.24, 0.2 cc., etc. and the volumes were adjusted to a total volume of 0.8 cc. before the addition of the inoculated red blood cell suspension).

The fact that, as will be shown in the present paper, penicillins F, G, K, and X are all inactivated by human and rabbit serum of necessity implies that the apparent penicillin content of a given serum, judged by a serial dilution technic such as that here used, will be in error because of a similar inactivation during the incubation of the assay tubes. This was clearly stated by Bigger in his important 1944 paper (6) and has usually been ignored, despite the fact that it vitiates the quantitative significance of penicillin assays at barely detectable levels. The magnitude of the error so introduced, which is particularly large with penicillin K, will be discussed in detail in a separate communication. In the present paper, the experimental data given in the tables, and the points plotted in the figures, are the apparent and uncorrected penicillin contents of the various specimens. The dotted curves in some of the figures are the assays corrected for this serum error. These corrections were usually negligible except in the case of penicillin K; and even with that penicillin, the correction was significant only when the serum specimens contained so little penicillin that only serum concentrations greater than 1:8 had demonstrable activity.

Penicillins.—We are indebted to the laboratories of the Upjohn Company, E. R. Squibb and Sons, Abbott Laboratories, and Lederle Laboratories, Inc. for their generous provision of the samples of crystalline F, G, K, and X, respectively, used in the present studies. With the exception of K, a single preparation of each penicillin was used throughout. In the case of K, two samples were used (Lots No. RP309P1 and RP309P2) which behaved indentically, and are not distinguished in the tables.

EXPERIMENTAL RESULTS

The Inactivation of Penicillins F, G, K, and X by Human and Rabbit Serum

A 1:25,000 aqueous solution of penicillins F, G, K, and X was diluted with 19 volumes of rabbit or human serum to give a 1:500,000 dilution in 95 per cent serum. This was incubated at 37°C. At varying intervals, aliquot portions were withdrawn and the inactivation reaction stopped by dilution with broth and storage at −10° to −20°C. (*cf.* page 154). The amount of active penicillin in the several stored samples was assayed simultaneously at the completion of the incubation period. As previously discussed the necessary presence of serum in the penicillin assays introduces a systematic error which was, however, quantitatively insignificant in the experiments of Figs. 1 and 2.

Illustrative experiments with rabbit and human sera are summarized in Figs 1 and 2. The results in a number of similar experiments with both human and rabbit sera are summarized in Tables I and II. In both human and rabbit sera, penicillin K was by far the most susceptible to inactivation, X was the

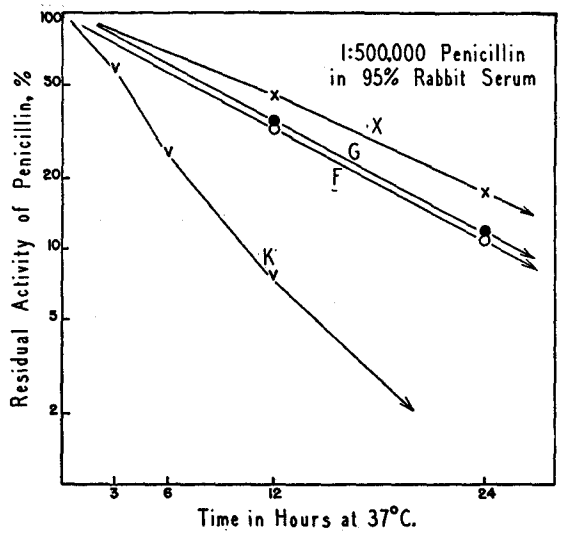

FIG. 1. The inactivation of penicillins F, G, K, and X by rabbit serum *in vitro* (Experiment of footnote 2, Table I). To a pooled rabbit serum was added 1/19th volume of a 1:25,000 dilution of crystalline penicillins F, G, K, and X, to give a 1:500,000 dilution of 95 per cent serum. Aliquot portions were removed at the intervals indicated in the figure (3, 6, 12, and 24 hours) and stored at −10° to −20°C. The residual penicillin was determined in all the samples simultaneously, at the end of the incubation period, using the corresponding penicillin diluted in broth as a reference standard. In this and all following figures, the arrow signifies that there was no demonstrable penicillin at the following time period tested.

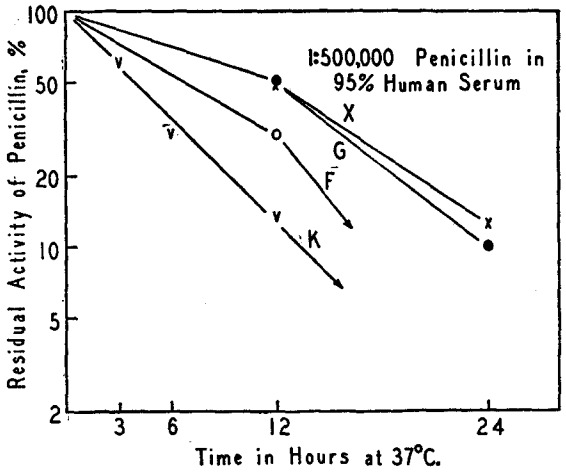

FIG. 2. The inactivation pf penicillins F, G, K, and X by human serum *in vitro* (Experiment V.O. of Table II). Penicillins F, G, K, and X in a final concentration of 1:500,000 were incubated at 37°C. in human serum (V.O.). Aliquot portions were removed at the time intervals indicated in the figure, stored at −10° to −20°C., and assayed simultaneously at the end of the incubation period.

least susceptible, with penicillins F and G intermediate. When a 1:500,000 dilution of penicillins F, G, K, or X in 95 per cent rabbit serum was kept at 37°C., the residual activity of penicillins F, G, and X had decreased in 24 hours to an average of 9, 7, and 19 per cent, respectively, while the solution of K then contained no demonstrable penicillin. In human sera also, the activity of F,

TABLE I

The Inactivation of Crystalline Penicillins F, G, K, and X in 1:500,000 Concentration (2 Micrograms per Cc.) by Rabbit Serum at 37°C.

Summary of series of experiments resembling those of Fig. 1.

Type of penicillin	Residual activity after incubation for 24 hrs. at 37° C. in															Average drop per hr.§
	Broth							Rabbit sera*								
	Individual experiments						Mean	Individual experiments							Mean	
								1	2	3	4	5	6	7‡		
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
F	72	75	75	90	92	90	82	9	10	15	4	5	—	11	9	9
G	80	61	77	100	80	75	78	—	11	13	‖	‖	4	12	7±	10.5
K	63	79	73	74	—	85	75	No demonstrable penicillin								18¶
X	67	80	75	90	83	73	78	27	22	28	11	—	—	18	19	7

* Each experiment with a different lot of serum pooled from 2 to 6 animals.

‡ Experiment of Fig. 1.

§ The figures in this column (per cent of residual penicillin destroyed each hour) were calculated from the 24 hour results, using the expression *activity at time t* = (*hourly residuum*) $^{\text{time in hours}}$. A penicillin activity of *e.g.* 9 per cent (0.09) after 24 hours gives an hourly residuum of 0.91; *i.e.*, 9 per cent of the residual penicillin is destroyed each succeeding hour.

‖ No demonstrable penicillin.

¶ Calculated from six 12 hour results, not given in the table, of 12, 5, 10, 11, 7, 8, and 11 per cent. The average of 9 per cent activity after 12 hours gives an hourly residuum of 82 per cent. This result has no absolute significance, for unlike penicillins F, G, and X, the rate of inactivation of penicillin K varies strikingly with its concentration (*cf.* page 150).

G, K, and X had fallen after 12 hours to an average of 30, 40, 11, and 51 per cent, respectively, and after 24 hours to 4, 9, < 0.6, and 16 per cent. Although not shown in the tables, with the occasional exception of X, there was no demonstrable activity in any of the serum-penicillin mixtures after 48 hours at 37°C. Control dilutions in broth similarly incubated retained 75 to 82 per cent of their original activity after 24 hours at 37°C.

In Figs. 1 and 2, as in most of the figures in this paper, the ordinate (residual penicillin in per cent) has been plotted on a logarithmic scale. It is apparent from the figures that this value (logarithm of residual penicillin) usually de-

creased at a constant rate[1] and that the rate of inactivation was therefore proportional to the amount of residual penicillin $\left(\frac{dx}{dt} = k(a - x)\right)$. The effect on this relationship of such factors as penicillin concentration, serum concentration, temperature, etc. will be discussed in following sections. Leaving these factors out of consideration, in whole rabbit serum at 37°C., the decrease in the con-

TABLE II

The Inactivation of Crystalline Penicillins F, G, K, and X in 1:500,000 Concentration (2 Micrograms per Cc.) by Human Serum at 37°C.

Summary of experiments resembling those of Fig. 2.

Penicillin species	Residual activity after incubation at 37°C. in																		Average hourly decrease in penicillin activity§	
	Broth* (24 hrs.)	Rabbit serum* (24 hrs.)	Human serum																	
			L.M. 24 hrs.	J.K.		J.		C.		L.M.		V.O.‡		U.K.		No. 248		Average		
				12 hrs.	24 hrs.	12 hrs.	24 hrs.	12 hrs.	24 hrs.	12 hrs.	24 hrs.	12 hrs.	24 hrs.	12 hrs.	24 hrs.	12 hrs.	24 hrs.	12 hrs.	24 hrs.	1st 12 hrs.	1st 24. hrs
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
F	82	9	4	19	‖	25	3	30	‖	30	‖	30	‖	—	—	46	25	30	4±	10	13
G	78	7	8	38	5	33	3	40	4	33	5	50	10	34	13	55	25	40	9	7	9
K¶	75	<1	‖	‖	‖	9	‖	10	‖	10	‖	13	‖	15	‖	17	‖	11	‖	17**	—
X	78	19	11	47	8	45	9	50	13	45	10	48	13	—	—	75	45	51	16	5	7

* Average of several experiments as indicated in Table I.

‡ Experiment of Fig. 2.

§ The figures in this column (per cent of residual penicillin destroyed each hour) were calculated from the 12 or 24 hour results, using the expression *activity at time t* = (*hourly residuum*)$^{\text{time in hours}}$. A residual activity of *e.g.* 4 per cent (0.04) after 24 hours gives an hourly residuum of 0.87; *i.e.*, 13 per cent of the residual penicillin is destroyed each succeeding hour.

‖ No demonstrable penicillin.

¶ Values given for penicillin K are the experimental data, not corrected for the serum error in the assay. That error is insignificant for penicillins F, G, and X (*cf.* page 142).

** This value is based on the 12 hour results. It has no absolute significance, for unlike penicillins F, G, and X, the inactivation of penicillin K varies strikingly with its concentration.

centration of residual active penicillin over a 24 hour period averaged 9, 11, and 7 per cent per hour[2] for F, G, and X, respectively. In human serum, the corresponding rates of fall were 13, 9, and 7 per cent per hour. Over a 12 hour period, the hourly inactivation of penicillin K averaged 18 and 17 per cent in rabbit and human serum.

[1] $t = \frac{1}{k} \ln \frac{a}{a - x}$, where t is time, a is the initial amount of penicillin, x is the amount inactivated in time t, and k is the velocity constant of the reaction.

[2] The amount remaining after 1 hour would thus be 91, 89, and 93 per cent; and the proportion of residual penicillin after t hours would be 0.91^t, 0.89^t, and 0.93^t for F, G, and X, respectively.

As will be discussed in a following section, the relatively high rate of K inactivation was further increased as the concentration of penicillin was reduced in both rabbit and human serum. The order of increasing susceptibility to inactivation by serum was X < F = G < K.

The Effect of the Concentration of Serum on the Inactivation of Penicillins F, G, K, and X.—An experiment to determine the effect of the serum concentration on the rate of inactivation of penicillin K is summarized in Fig. 3. Quantitatively similar results in a number of experiments with both human and rabbit serum are summarized in Table III. In order to simplify the presentation of the experimental data, the table lists only the time required to inactivate 90 per cent of the penicillin, obtained by appropriate interpolation on a curve resembling those of Fig. 3.

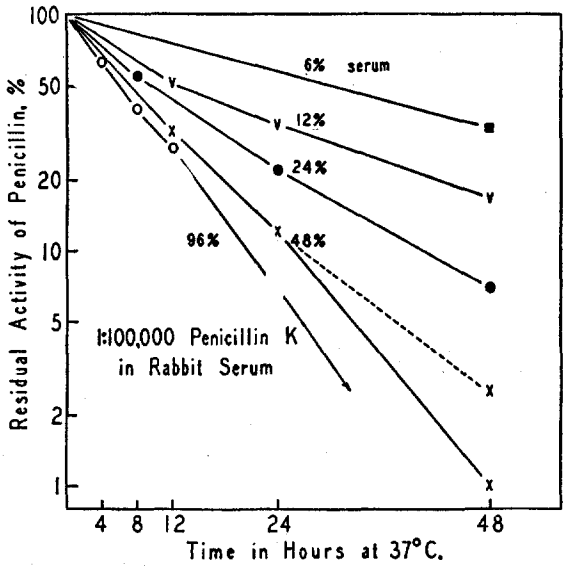

FIG. 3. The effect of the concentration of serum on the inactivation of penicillin K at 37°C. To a pooled serum was added 1/24th volume of 1:4000 penicillin K to give a 1:100,000 dilution in 96 per cent serum. This was serially diluted with a 1:100,000 dilution of the same penicillin in broth, and all the tubes were simultaneously incubated at 37°C. At the intervals indicated in the figure (4, 8, 12, 24, and 48 hours) aliquot samples were removed and stored at −10° to −20°C., preparatory to simultaneous assay at the conclusion of the incubation period. As in all the figures, the arrow signifies that there was no residual penicillin at the next time period tested. The dotted line indicates the percentage of residual penicillin corrected for the serum error in the assay, and is the only point in the figure in which that error was significant.

It is apparent in Fig. 3 and Table III that the rate at which penicillins F, G, K, and X were inactivated by human or rabbit serum varied with the serum concentration. In the case of penicillins F, G, and X, the relationship was

precisely linear, in that the time required to inactivate a given proportion of the penicillin, *e.g.*, 90 per cent, varied directly with the concentration of serum.

TABLE III

The Effect of the Concentration of Human and Rabbit Serum on the Inactivation of Penicillins F, G, K, and X

One volume of 1:20,000 penicillin solution was added to 24 volumes of serum to give a 1:500,000 dilution in 96 per cent serum. This was serially diluted with a 1:500,000 dilution of the same penicillin in broth, and all the tubes were simultaneously incubated at 37° C. Aliquot samples were removed at intervals (*e.g.* 3, 6, 12, 24, and 48 hours) and stored at −10° to −20° C., preparatory to simultaneous assay at the conclusion of the incubation period. In Experiment 3 with human serum, and Experiment III*a* with rabbit serum, the final concentration of penicillin was 1:2,500,000 instead of 1:500,000 and in Experiment II, which is the experiment of Fig. 3, the final dilution of penicillin was 1:100,000.

Serum	Penicillin species	Experiment No.	Concentration of serum in reacting mixture, *per cent*				
			96	48	24	12	6
			Time required to inactivate 90 per cent of penicillin				
			hrs.	*hrs.*	*hrs.*	*hrs.*	*hrs.*
Human	F	2	40	75*	128*		
	G	2	40	85*	168*		
	K	1	16	24	28	38	115*
		2	16	19	38	56	—
		3	5	—	18	—	
		4	18	21	38	52	
	X	2	60	128*	—	—	—
Rabbit	F	III	25	58	112*		
	G	III	27	48	94*		
	K	I	5	9	20	33	
		II‡	21	26	38	63	
		III	10	13	22	50	96*
		III*a*	3	—	16	—	
		IV	11	14	21	56	
	X	III	38	80	160*		

* Approximation only obtained by extrapolation of the data beyond the experimental period of 48 hours.

‡ Experiment of Fig. 3.

This is evident in Table III, and is graphically shown in Fig. 4, in which are plotted the results of a single experiment (human serum III of Table III).

For penicillins F, G, and X, one may therefore write the inactivation reaction as $t = \frac{1}{kS} \ln \frac{a}{a - x}$, where t is the time in hours, k is a velocity constant, S is the serum concentration, a is the initial concentration of penicillin, and x is the amount inactivated in t hours. This relationship did not hold for penicillin

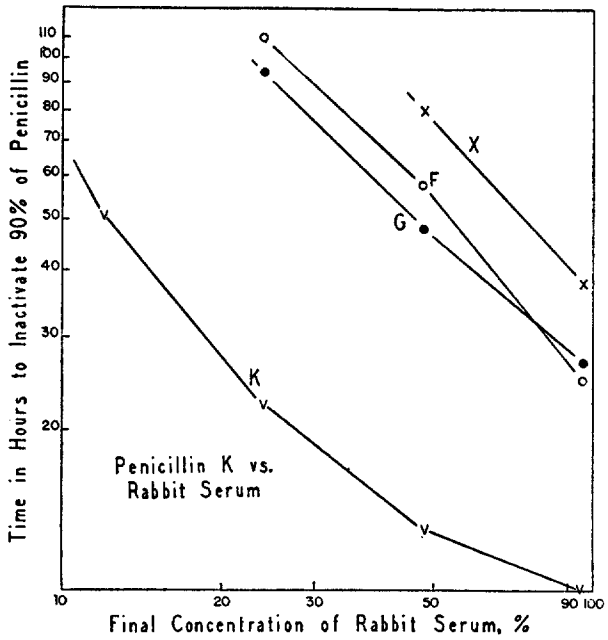

FIG. 4. The effect of the concentration of serum on the rate of inactivation of penicillins F, G, K, and X (Experiment III of Table III). Final concentration of penicillin 1:500,000 throughout. Each point in the figure is based on a curve such as those of Fig. 3. Inactivation times of greater than 50 hours are estimated values obtained by the extrapolation of such experimental curves. Because the inactivation regularly proceeded at a logarithmic rate, that extrapolation is believed to be reasonably reliable.

K, with which the inactivation time was not linearly related to the serum concentration.

The observed experimental data with respect to the inactivation of penicillins F, G, and X by human and rabbit serum can therefore be explained on the basis of a bimolecular interaction between penicillin and an as yet unidentified serum factor, with the latter present in such large excess under the conditions of the experiment that the kinetics of the reaction are those of a pseudo first order reaction.

The quantitative differences between the susceptibility of penicillins F, G, K, and X to inactivation by serum indicated in Tables I and II are again apparent

in Table III and Fig. 4. Penicillin X was the least susceptible; F and G were again intermediate; and K was by far the most susceptible. By and large, a 1:8 dilution of either rabbit or human serum was just as active against K (30 to 56 hours required for 90 per cent inactivation of a 1:500,000 dilution) as whole serum against F, G, or X (25 to 60 hours for a corresponding degree of inactivation).

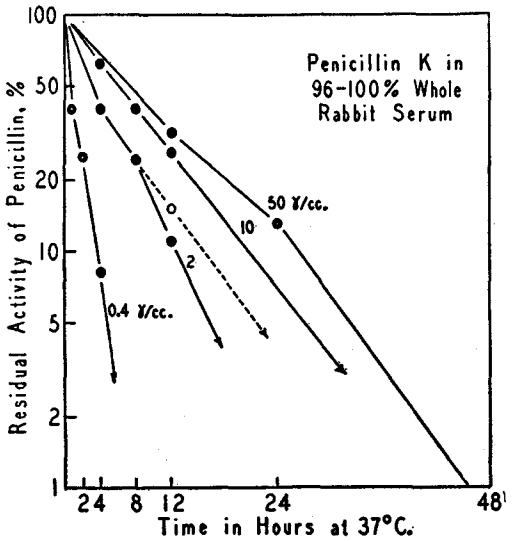

FIG. 5. The effect of the concentration of penicillin K on the rate of inactivation by rabbit serum. To a pooled rabbit serum was added 1/19th volume of a 1:1000 solution of penicillin K to give a 1:20,000 dilution in 96 per cent serum. This was serially diluted with the same serum to give dilutions of 1:100,000, 1:500,000, and 1:2,500,000 in 99.1 to 99.9 per cent serum. Aliquot portions were removed at the intervals indicated in the figures and stored at −20°C. preparatory to simultaneous assay. The arrows and dashed lines have their usual significance (*cf.* legend to Fig. 3). The open circle and dotted line indicate an experimental value corrected for the error caused by the presence of serum in the assay. That correction was insignificant for the other points in that curve, and at higher concentrations of penicillin. The experiment with 0.4 microgram per cc. is subject to an even larger correction. This is, however, not indicated in the figure because it is quantitatively indeterminate.

Effect of Penicillin Concentration on Rate of Inactivation.—Of practical interest was the degree to which the rate of inactivation of penicillins F, G, K, and X by serum varied with the penicillin concentration, and in particular, how rapidly these penicillins were affected at the concentration levels attained in the course of their therapeutic administration.

An illustrative experiment with penicillin K in rabbit serum is summarized in Fig. 5. In general, the lower the concentration of penicillin K, the faster it was inactivated. Thus, in the experiment of Fig. 5, in the (therapeutically)

high concentration of 50 micrograms (115 "units") per cc., it required 26 hours to inactivate 90 per cent of the penicillin. At concentrations of 10 and 2 micrograms per cc., the 90 per cent inactivation period was 22 and 13 hours, respectively; and at a level of 0.4 microgram (0.9 unit) per cc., effectively bactericidal for most organisms susceptible to penicillin, more than 90 per cent of penicillin K was apparently inactivated in 4 hours. The quantitative significance of this last value is, however, dubious in view of the large error introduced by the high concentration of serum necessarily present in the penicillin assays (*cf.* page 142). In the other experiments, however, save for the one dotted line in Fig. 4, this serum error was insignificant.

A number of similar experiments with penicillins F, G, K, and X in both human and rabbit sera are summarized in Table IV, in which are given only the times required to inactivate 90 per cent of the penicillin. These were obtained by appropriate interpolation on curves such as those of Fig. 5. To clarify presentation, the results with penicillin K are graphically shown in Fig. 6.

It is apparent that the rate of inactivation of penicillin K increases as its concentration is reduced. In the experiments of Table IV, the average time required for 90 per cent inactivation in rabbit serum fell from 23 to 13 to 9 to 3 hours as the concentration was reduced from 50 to 10 to 2 to 0.4 micrograms per cc. These values correspond to an hourly decrease of 9, 16, 21, and 50 per cent, respectively. In human serum, as the concentration of K was reduced from 50 to 2 to 0.4 micrograms per cc., the inactivation time fell from (approximately) 21 to 15 to 3 hours, corresponding to hourly rates of fall of 10, 14, and 50 per cent, respectively. The results at the lowest penicillin concentration have no quantitative significance because of the relatively large serum error but the qualitative trend is clear. One could reasonably anticipate that at even lower concentrations, still effectively bactericidal, penicillin K would be inactivated by serum so quickly as to be therapeutically inert. This cannot be shown experimentally because the inhibitory effect of serum precludes the assay of penicillin K at these low concentrations (*cf.* page 142).

In marked contrast to penicillin K, the rate of inactivation of penicillins F, G, and X was largely independent of their concentration throughout the range 0.4 to 50 micrograms per cc. This is shown in Table IV, and more clearly in Fig. 7, which is to be contrasted with Fig. 6. Penicillin X was consistently inactivated more slowly than were F or G by the same serum; but whether in rabbit or human serum, the rate of inactivation of F, G, and X was largely independent of their concentration. In most of the experiments, the time required to inactivate 90 per cent of the penicillin was exactly the same at 50 micrograms per cc. as it was at 0.4 microgram per cc., and the maximum time difference observed over this more than 100-fold difference in penicillin concentration was less than twofold.

TABLE IV

The Effect of the Concentration of Penicillins F, G, K, and X on their Inactivation by Human Serum at 37°C.

Serum	Penicillin species	Dilution of penicillin	Serum No. 1	Serum No. 2	Serum No. 3	Serum No. 4
			Time for 90 per cent inactivation			
			hrs.	*hrs.*	*hrs.*	
Human	F	20,000	21	—	31	
		100,000	18	39	—	
		500,000	18	—	40	
		2,500,000	15	30	32	
	G	20,000	25	—	35	
		100,000	24	43	—	
		500,000	24	—	40	
		2,500,000	29	42	33	
	K	20,000	16	—	27	
		100,000	17	27	—	60
		500,000	13	—	16	16
		2,500,000	1½	6	5	1
	X	20,000	29	—	54	
		100,000	26	60	—	
		500,000	26	—	60	
		2,500,000	20	48	80	
Rabbit	F	20,000		—	28	
		100,000		36	—	
		500,000		—	25	
		2,500,000		24	17	
	G	20,000		—	27	
		100,000		28	—	
		500,000		—	27	
		2,500,000		29	19	
	K	20,000	27	—	20	—
		100,000	22	7	—	11
		500,000	13	—	10	5
		2,500,000	3½	2½	3	—
	X	20,000		—	48	
		100,000		68	—	
		500,000		—	38	
		2,500,000		72	42	

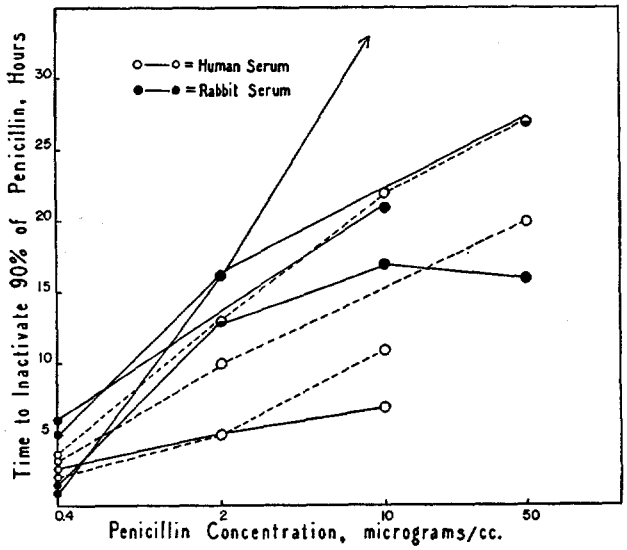

FIG. 6. The rate of inactivation of penicillin K in rabbit and human serum as a function of penicillin concentration (from data of Table IV).

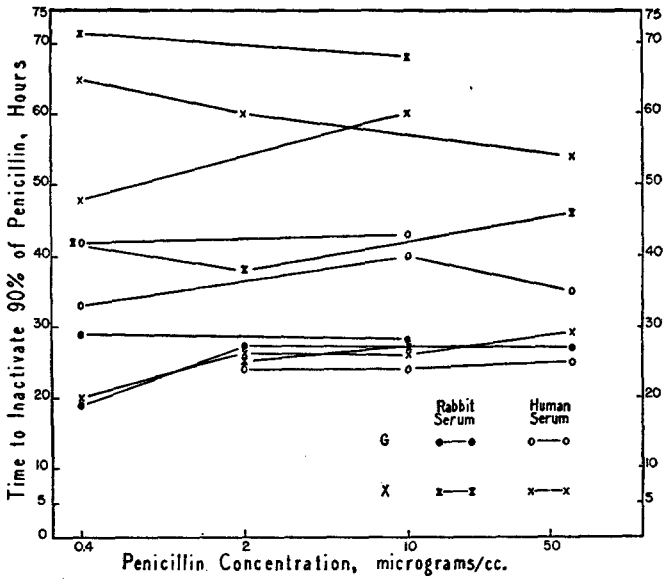

FIG. 7. The inactivation of penicillins G and X by human and rabbit serum, and its independence of the penicillin concentration. Each point in the figure is based on a curve such as those of Fig. 5. Inactivation times of greater than 50 hours are estimated values obtained by the extrapolation of such experimental curves. Because the inactivation regularly proceeded at a logarithmic rate, that extrapolation is believed to be reasonably reliable.

The inactivation of penicillins F, G, and X by serum therefore behaves like a first order reaction, in that the time required to inactivate a given proportion of the penicillin by a fixed concentration of serum is largely independent of its original

TABLE V

The Rate of Inactivation of Penicillins F, G, K, and X by Human and Rabbit Serum at 37°C.

Serum	Penicillin species	Dilution of penicillin	Serum No. 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Mean	Average destroyed per hr.*	Average velocity constant $\left(k = \frac{1}{t} \times \ln \frac{a}{a-x}\right)$
			Time required to inactivate 90 per cent of penicillin																	
			hrs.	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*per cent*	*hrs.*$^{-1}$
	F	§	23	24		18		17	19	23	23	18‖	35‖			34‖		23	9.5	1.0×10^{-1}
	G	§		25		16		20	18	19	20	24‖	43‖	28		36‖		22	10	1.1×10^{-1}
Human		20,000										16				27		21±	10.4	1.1×10^{-1}
		100,000										17	27			16		20±	11	1.15×10^{-1}
	K	500,000			16		17		13	12	12	13		15	20		18	15	14.3	1.5×10^{-1}
		2,500,000			<1							1½	6			5		3±	50±	$7.7\pm \times 10^{-1}$
	X	§	42	37		26		22	23	27	24	25‖	54‖			61‖		34	6.5	0.7×10^{-1}
	F	§	29	18				30‖			23‖							25	9	0.9×10^{-1}
	G	§	30	24		31		29‖	19		24‖							26	8.5	0.9×10^{-1}
Rabbit		20,000				27					20							23	9.5	1.0×10^{-1}
		100,000			11	22		7										13	16.0	1.8×10^{-1}
	K	500,000			5	13	8		12	13	10							10	20.5	2.3×10^{-1}
											11									
		2,500,000				3½		2½			3							3±	50±	$7.7\pm \times 10^{-1}$
	X	§	43	28				70‖			42‖							46	5.0	0.5×10^{-1}

* Since the penicillin activity falls off logarithmically, a residual activity of 10 per cent after *e.g.* 23 hours (first value in preceding column) implies an hourly residuum x given by the equation $x^{23} = 0.1$, or 0.905; *i.e.*, 1 — 0.905 = 9.5 per cent of the residual penicillin is destroyed each succeeding hour.

‡ a = original penicillin, x = amount destroyed in t hours. For 90 per cent inactivation, $\ln \frac{a}{a-x} = 2.3026$; an inactivation time of *e.g.* 23 hours gives a velocity constant of 0.1.

§ All tested at 1:500,000 concentration except that indicated by ‖.

‖ Average of results at concentrations varying from 1:20,000 to 1:2,500,000 (*cf.* Fig. 7 and Table IV).

concentration $\left(t = \frac{1}{k} ln \frac{a}{a-x}\right)$. By ignoring the unknown factor introduced by the concentration of the reactive serum substance, and incorporating that unknown factor in the velocity constant, one obtains the average "constants" given in the last vertical column of Table V for the inactivation of penicillins F, G, and X by rabbit and human serum at 37°C.

In both rabbit and human serum, X was inactivated more slowly than peni-

cillins F or G, with velocity constants of 0.07 to 0.05, as compared with 0.09 to 0.11. The velocity constant for the inactivation of K varied with its concentration. At concentrations of 50 micrograms per cc. the rate of inactivation was precisely the same as that of F, G, or X, with a percentage loss of 9 and 10 per cent per hour in human and rabbit serum, respectively, and velocity constants of 0.1 and 0.11. The rate of inactivation increased progressively as its concentration was reduced. This suggests that K may be inactivated by two distinct mechanisms, one peculiar to itself, and demonstrable only at low concentrations of penicillin, and one identical with that affecting F, G, and X.

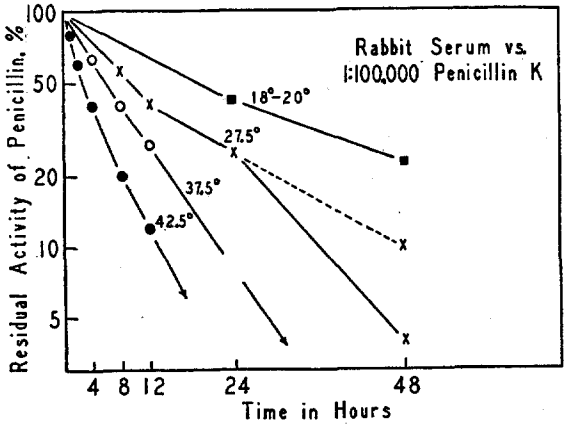

FIG. 8. The effect of temperature on the inactivation of penicillin K by rabbit serum. As in all the figures, the arrow signifies that there was no demonstrable penicillin at the next time period tested. The dotted line refers to an experimental value corrected for the error introduced by the presence of serum in the assay, and is the only point in the figure for which that error was significant.

This possibility is further supported by the experimental data of the following sections.

The Temperature Coefficient of the Serum Inactivation of Penicillin.—The effect of temperature on the serum inactivation of penicillin is illustrated in Fig. 8. The higher the temperature, the more rapidly was K inactivated. At 42.5°, 37.5°, 27.5°, and 20°C., it required 13, 22, 48, and 90 (estimated) hours, respectively, for a pooled rabbit serum to inactivate 90 per cent of 1:100,000 K (Fig. 8); and in another experiment with a pooled human serum, at 42.5°, 37.5°, 32.5°, and 27.5° it required 9, 15, 25, and 35 hours to inactivate 90 per cent of a 1:500,000 solution. At ice box temperature there was no demonstrable inactivation even after 48 hours. Control dilutions of penicillin in broth showed only a slight loss in activity after 24 hours at 42.5° (residual activity of 80 per cent in a 1:100,000 solution, and 60 per cent in a 1:500,000 solution).

If the logarithm of the time required to inactivate 90 per cent of penicillin K (a value which is proportional to the logarithm of the velocity constant) is plotted against the temperature, the data of three individual experiments fall reasonably well along a straight line (Fig. 9). So measured, the temperature coefficient for the inactivation of penicillin K by human or rabbit serum is approximately 2.5 for each 10°C.

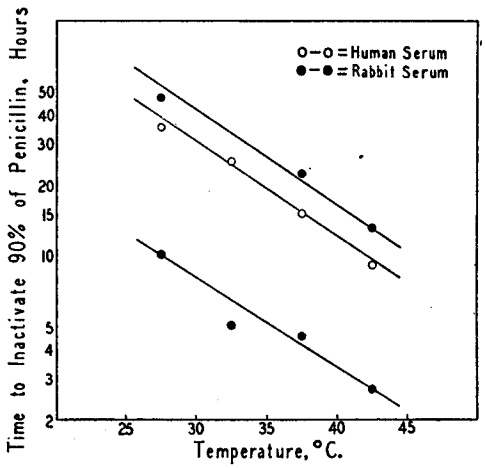

FIG. 9. The temperature coefficient for the inactivation of penicillin K by human and rabbit serum. The ordinates are the times required to inactivate 90 per cent of the penicillin, obtained by graphic interpolation on curves such as those of Fig. 8. Three experiments are shown, two with rabbit sera, and one with a human serum. The large difference in the absolute rate of inactivation in the two experiments with rabbit sera reflects the varying initial concentration of penicillin K (1:100,000 and 1:500,000). The human experiment was carried out with 1:500,000 penicillin. The straight lines in the figure all have a slope corresponding to a temperature coefficient of 2.5 for each 10° difference in temperature.

Like penicillin K, penicillins F, G, or X were inactivated to only a minor degree in 24 hours at 20°C. Thus, when 1:500,000 solutions of penicillins F, G, K, and X were incubated in a human serum, the residual activities after 24 hours at 20°C. were 45, 67, 38, and 70 per cent, respectively; and in a similar experiment with rabbit serum, the residual activities after 24 hours at 20°C. were 60, 42, 44, and 70 per cent for F, G, K, and X, respectively.

The Thermolability of the Penicillin Inactivator in Serum.—Since the time required for the inactivation of penicillins F, G, or X varies directly with the concentration of the serum factor, the inactivation time may be taken as a direct measure of the serum activity. So measured, the substance in human and rabbit serum responsible for the slow inactivation of penicillins F, G, or X proved to be relatively thermostable, and was usually unaffected by heating at 56°C., for 30 to 60 minutes (*cf.* Fig. 10), the maximum loss in activity observed being on the order of 10 to 20 per cent.

In contrast to these results, the serum substance responsible for the rapid inactivation of K was relatively thermolabile. In both human and rabbit serum, its activity was demonstrably reduced in 5 to 10 minutes at 56°C. (Fig. 11). Such heated sera had the same slight effect on K as they did on F,

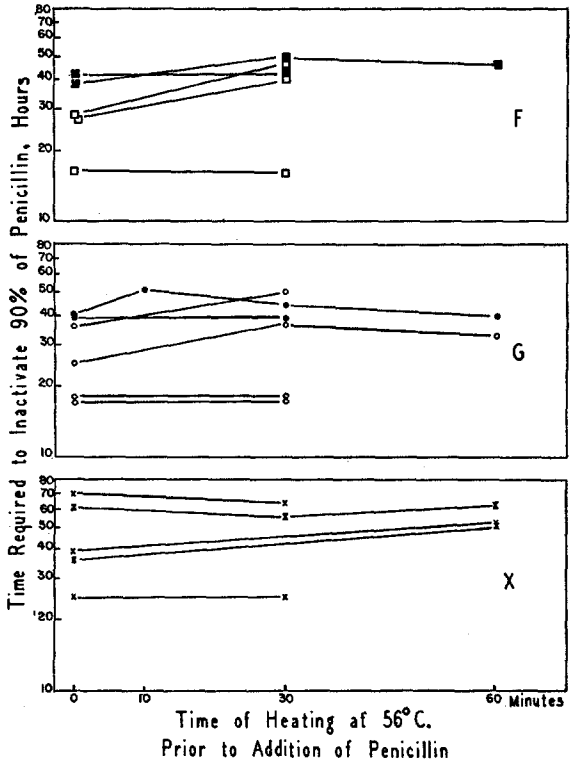

FIG. 10. The thermostability (at 56°C.) of the F-, G-, and X-inactivating agent in rabbit and human sera. Solid points refer to rabbit sera, and open points to human sera. Inactivation times of greater than 50 hours are estimated values extrapolated from experimental curves such as those of Figs. 3 or 5.

G, or X, and that slight residual activity was not significantly affected by further heating up to 60 minutes at 56°C.

In order to determine the degree to which the K-inactivating factor had been destroyed by heating at 56°C., the effect of such heated serum was compared with that of the same fresh serum diluted 1:2, 1:4, 1:8, and 1:16 in broth. An illustrative experiment is shown in Fig. 12; and a number of similar experiments with both human and rabbit sera are summarized in Table VI. The values given in the table are the times required for 90 per cent inactivation obtained by graphic interpolation on a curve like those of Fig. 12. Seventy-

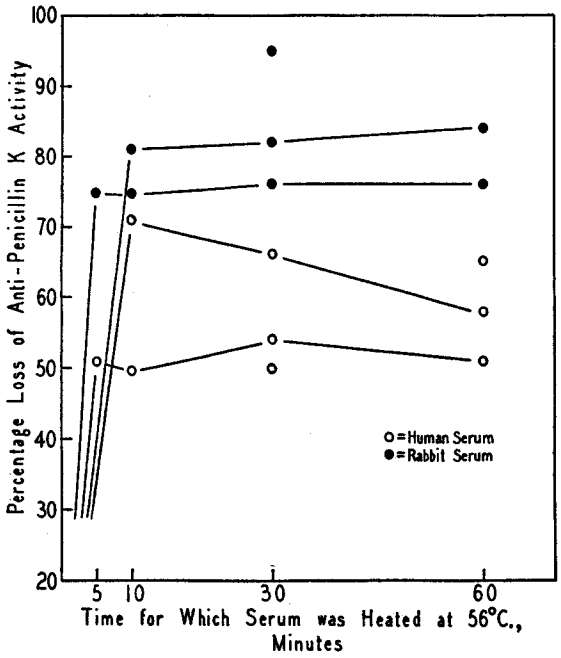

FIG. 11. The thermolability of the K-inactivating agent in rabbit and human sera.

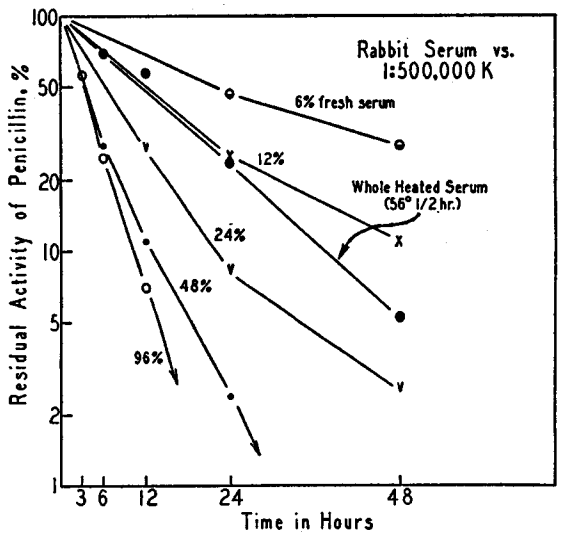

FIG. 12. The relative activity of heated and fresh rabbit sera on 1:500,000 penicillin K. Penicillin was incubated in varying dilutions of fresh rabbit serum as indicated in the figure. Simultaneously, whole (96 per cent) rabbit serum was tested which had previously been heated at 56°C. for ½ hour. As is apparent in the figure, the heated serum was approximately 15 per cent as active as the fresh serum. None of the points in the figure have been corrected for the error introduced by the presence of serum in the penicillin assays.

five to 80 per cent of the K-inactivating principle of rabbit serum was destroyed in 5 to 10 minutes at 56°C.; the corresponding loss in human serum was an average of 57 per cent. The residual activity was relatively thermostable, and was of the same order of magnitude as the activity of the fresh serum against F, G, or X (compare Figs. 10 and 11).

These observations, in conjunction with those of the preceding section, suggest that serum has two qualitatively different effects on penicillin: a slow inactivation of penicillins F, G, K, and X by a thermostable serum component, and a much more rapid inactivation of penicillin K alone by a relatively thermolabile agent, demonstrable only in low concentrations of penicillin.

TABLE VI

The Effect of Heating at 56°C. on the K-Inactivating Agent of Rabbit and Human Serum

Serum	Experiment No.	Fresh serum, *per cent*				96 per cent serum, heated at 56° C. for				96 per cent serum, heated at 56° C. for			
		96	48	24	12	5 min.	10 min.	30 min.	60 min.	5 min.	10 min.	30 min.	60 min.
		Time required to inactivate 90 per cent of penicillin				Time required to inactivate 90 per cent of penicillin				Loss in activity			
		hrs.	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*per cent*	*per cent*	*per cent*	*per cent*
Human	1	16	24	28	38			24				50	
	2	16	19	38	56		36	31	25		71	66	58
	3	5		18					16				65±
	4	18	21	38	53	22	21	24	22	51	50	54	51
Rabbit	I	5	9	20	33			70				95±	
	II	10	13	22	50		35	37	41		81	82	84
	III	11	14	21	56	22	24	26	26	75	75	76	76

The Inactivation of Penicillin by Serum, Plasma, and Red Blood Cells.—As is illustrated in Fig. 13, human serum and heparinized plasma were identically active in the inactivation of penicillins G or K. An incompletely washed 40 per cent suspension of red blood cells in heparinized salt solution had only a slight effect. Finally, whole heparinized blood was regularly less active than serum or plasma, presumably by virtue of its inactive red blood cell fraction.

Not only was the penicillin not inactivated by the red blood cells, but as found by Rammelkamp and Keefer (4), it did not distribute itself equally between the cells and the surrounding fluid. With carefully washed cells, even after 24 hours at 37°C., 75 per cent of the original penicillin was recoverable in the surrounding fluid, as compared with 80 to 90 per cent in broth controls containing no cells.

Qualitatively similar results were obtained in rabbit serum, plasma, and whole blood. Serum and plasma were identically active, and slightly more

active per cubic centimeter than whole blood. The difference was referable to the fact that the red blood cells behaved as inert suspended material into which the penicillin did not diffuse, giving a higher initial concentration of penicillin in the surrounding fluid.

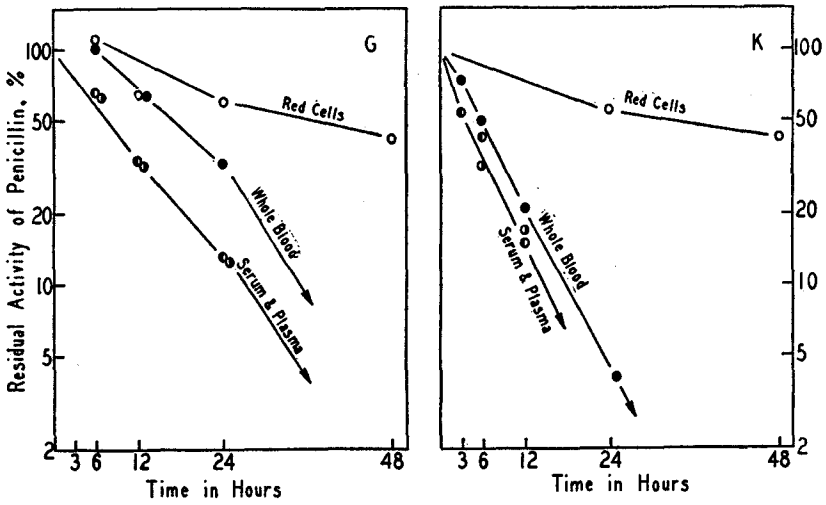

FIG. 13. The relative activity of human serum, plasma, whole blood, and washed red blood cells against penicillins G and K. One-nineteenth volume of 1:25,000 penicillin was added to one volume of (*a*) serum, (*b*) heparinized plasma, (*c*) heparinized whole blood, and (*d*) washed 50 per cent suspension of red blood cells (two washings with 1.5 volumes of heparinized salt solution). At the intervals indicated in the figure, aliquot samples were withdrawn and frozen at −25°C. (red blood cells removed by rapid centrifugation of the chilled specimens). All the samples were assayed simultaneously. In calculating the percentage of residual penicillin, the initial concentration was taken as 1:500,000, ignoring the fact that penicillin does not distribute itself equally between plasma and red blood cells. In consequence, the actual initial concentration in the free fluid of the whole blood and red blood cell suspension was almost twice that in serum or plasma, explaining the apparently slower inactivation observed.

SUMMARY AND DISCUSSION

1. Penicillins F, G, K, and X were all inactivated by human and rabbit serum, but two qualitatively distinct mechanisms were apparently involved.

2. One was a slow inactivation of all four penicillins by a relatively thermostable serum component which was not demonstrably affected by heating for 60 minutes at 56°C.

(*a*) In both human and rabbit serum this general inactivation of penicillin behaved like a pseudo first order reaction, with a velocity constant of 0.05–0.07 for penicillin X, and 0.09–0.11 for penicillins F and G.

(*b*) The percentage of penicillins F, G, and X inactivated per hour was independent of their concentration over the range 0.4 to 50 micrograms per cc.,

averaging 9.5, 10, and 6.5 per cent, respectively, in human serum, and 9, 8.5, and 5 per cent in rabbit serum.

(*c*) The rate of inactivation varied linearly with the concentration of the serum factor.

(*d*) Penicillin X was consistently and significantly less susceptible to inactivation than any of the other penicillins. Although minor differences were observed between F and G, these were not consistent, and are of questionable significance.

3. Superimposed on this slow inactivation of penicillins F, G, K, and X by a thermostable serum component was a much faster inactivation observed only with penicillin K.

(*a*) In both rabbit and human serum, the serum factor responsible for this inactivation was highly thermolabile, and was almost completely destroyed within 5 minutes at 56°C., leaving only a thermostable component, not affected by further heating.

(*b*) The inactivation of K by this thermolabile component was not a first order reaction, but varied with the concentration of both serum and penicillin. At high concentrations of K, the rate of inactivation due to the thermolabile factor was negligible, and penicillin K was destroyed no more rapidly than F, G, or X. The rate of inactivation increased as the concentration of penicillin was reduced. At penicillin K concentrations of 50, 10, 2, and 0.4 micrograms per cc., the hourly destruction in rabbit serum averaged 10, 16, 21, and 54 per cent. The corresponding figures in human serum were 10, 11, 14, and 54 per cent. The reservations entailed by the large serum error at the lower concentrations of penicillin are discussed in the text.

4. The temperature coefficient for the inactivation of penicillin K by fresh human or rabbit serum was 2.5 for each 10°C. No significant inactivation was observed in 24 hours at 20°C.; and this was true also of penicillins F, G, and X.

5. Heparinized plasma was just as active as serum, washed red blood cells had no effect, and the activity of whole blood was referable to its plasma content.

6. The nature of the serum factors responsible for these two types of penicillin inactivation are under present study.

7. The urinary excretion of penicillin is so rapid that the slow destruction of penicillins F, G, and X in the circulating blood as here described is of secondary significance therapeutically. It nevertheless must contribute to their rapid disappearance from the blood; and the fact that X is inactivated more slowly than either F or G could be reflected in higher and more sustained blood levels than are afforded by the latter two species. There are some reports that such is the case (15–17), and the following paper provides further evidence for the superiority of penicillin X in this respect over the other species so far studied.

The serum inactivation of penicillin K, at a rate which increases as its con-

centration falls, should be reflected in significantly lower and more evanescent blood levels than are observed with penicillins F, G, or X. As will be discussed in the following paper, this has been found to be the case, and provides a simple explanation for its paradoxically low therapeutic activity *in vivo* (8–11).

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