A STUDY OF THE TOXIC PROPERTIES OF TUBERCULO-PROTEINS AND POLYSACCHARIDES

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It was reported by White in 1928 (1) and confirmed by Sabin, Doan, and Forkner (2, 3) that a polysaccharide isolated from the tubercle bacillus by Anderson (4) was toxic for tuberculous guinea pigs.

With doses of 10 mg. the guinea pig either died within a few hours, in which case there was a precipitous fall in temperature; or it survived and showed only a temporary fall in temperature with subsequent rise above the normal level. There was also a change in the blood cells characterized by a rise in the neutrophilic leucocytes and a fall in lymphocytes.

Similar phenomena follow the administration of tuberculo-protein. Since the polysaccharide, as prepared, has a certain nitrogen content, and since all the protein preparations contain carbohydrate, one must resort to a biological titration in order to determine responsibility for the intoxication. By giving the polysaccharide and the protein to tuberculous animals, in decreasing doses, we have ascertained that the temperature reaction is due to the protein, since amounts of polysaccharide which do not contain as much nitrogen as is present in a temperature-altering dose of the protein fail to elicit the change.

In the course of our testing of fractions from tubercle bacilli, it has become clear that we must consider not only fractions from different types of bacilli, according to the plan of the Research Committee of the National Tuberculosis Association (5), but also the varying manifestations of the disease in different species of animals. To show the relations of these different factors we have drawn up an outline (Table I). The attempt at any quantitative comparative estimation of the factors of the disease must be considered as only an approximation, subject to marked variations in individual animals. For the

TABLE I

Phenomena of Tuberculosis as Related to Chemical Fractions from the Bacilli

Phenomena of the disease		Production of lesions: Tubercles	Production of lesions: Non-specific connective tissue	M/L index in blood	Rate of growth of bacilli	Estimated proportion of dead to living bacilli	Change of virulence of bacilli as from S to R	Acute and subacute or chronic	Loss in weight	Acute toxic death (experimental)	Allergy (skin test)	Caseation	Temperature	Hemorrhage
Relation of chemical fractions from the bacilli to these phenomena		Phosphatide	Wax and protein	Relation of phosphatide to anti-phosphatide	?	?	?	?	?	Protein and polysaccharide	Protein	Probably protein	Protein	Protein
Animal	Type of bacillus													
Fowls	Avian S (Petroff)	+		Reversed ++++	++++	Small		Acute	++++		±	−		
	Avian R (Petroff)	+++		Slight change	++	Average		Subacute	++		++++	++		
Guinea pigs	H_{37}	+++		Reversed ++	Average	Average		Subacute and chronic	Minimal	Protein 10–2.5 mg. and polysaccharide	++++	+++	Protein up to .0001 mg.	++++ Lymph nodes
Rabbits	B_1 and fractions of H_{37}	++++	++++ ++	Reversed ++++	Average	Large		Acute or chronic	+++		+	++ Slight with phosphatide in normal		++
Monkey		++++	++++	Reversed ++++	++++	Large		Acute	±		±	++++	Sensitive to O. T.	++++
Rat		++++				Extreme		Chronic	+		−	− Epithelioids filled with fat		++

Along the upper transverse line of the table are listed the phenomena of the disease itself and in the second line are recorded the effects of chemical fractions isolated from tubercle bacilli on normal and tuberculous animals as far as they are known. The lower part of the chart records the varying manifestations of the disease in animals, viewed in the light of studies with chemical fractions from the bacilli.

data on the temperature reactions in the monkey we are indebted to Dr. G. P. Berry whose work will soon be published.

The phase of the subject upon which we have done the most intensive work (3) is that of the chemical factors causing the production of new tissue. Both lipoids and proteins in repeated doses have given rise in rabbits to the production of new connective tissue cells, but a phosphatide designated A-3 by Anderson (6) has proved to be so specific in the production of epithelioid cells and epithelioid giant cells, the essential elements of the tubercle, that we may speak of this element in the disease as the phosphatide reaction. In the tuberculous animal the phosphatide is probably liberated only from the bodies of dead bacilli. The amount of the reaction to the phosphatide is governed by two different factors, first, by the number of dead bacilli, which can only be estimated; and second, by the amount of antiphosphatide produced in the disease (7, 8, 9).

The most striking new material shown in the table is represented by the preliminary analysis of the effects of the dissociation of the avian strain of bacilli by Petroff (10, 11, 12). These two strains were given to us by Dr. Petroff for pathological studies. As shown in Table I, the two dissociated strains of the bacilli produce different types of tuberculosis, that is, two different combinations of the manifestations of the disease. Roosters with the avian S strain had no tubercles large enough to be seen with the unaided eye, but under the microscope there were numerous tiny clumps of pure epithelioid cells, containing very large numbers of tubercle bacilli. Smears stained for tubercle bacilli had too many organisms per oil immersion field to be counted. From the small phosphatide reaction, we estimate that there had been a relatively slight death rate of bacilli. The animals had negative skin tests but became extremely emaciated.

With the avian R strain, on the other hand, the skin test was marked and the loss in weight only nominal, while tubercles were comparable in size to those seen in rabbits. Such striking differences in reaction to bacterial dissociation demonstrate the significance of this method in the further study of tuberculosis. A comparison of the reaction with the avian S in roosters with the disease in rats is also interesting. Both show a large number of bacilli, but in the case of the rat, we estimate an increased death rate of bacilli from the extent of the phosphatide reaction, the rat finally dying from the amount of epithelioid cells in the lungs rather than from any toxic effect. The detailed account of the pathological differences with dissociated strains will be published subsequently.

The data in Table I suggest, for the further testing of chemical fractions, the selection of animals showing certain extreme reactions, such as the loss of weight after the Petroff avian S in roosters, or the caseation in monkeys. The hypersensitivity of the guinea pig to tuberculo-protein has been the reason that this animal has been used so extensively for the study of allergy. Through the studies reported

here, it seems probable that the temperature in tuberculosis as indicated in Table I, is related to proteins liberated from the bacilli. The tuberculo-proteins give a rise in temperature in the normal animal but the reaction is increased in the sensitized animal.

The two types of temperature curves, one the fall which precedes death, and the other the reaction from which the animal recovers, are shown in Charts 1 to 7 for both proteins and polysaccharides. The results of the titrations of these two substances in tuberculous guinea pigs are shown in Tables II and III.

Most of the experiments with the protein have been made with a preparation from the H. K. Mulford Company, designated MA-100, which gives an active skin test in tuberculous guinea pigs in doses of 0.0001 mg. One guinea pig received the Protein 304 and another an alkali-soluble protein, both prepared from the bacilli by Johnson, Coghill and Renfrew (13, 14).

Four preparations of tuberculo-polysaccharides have been used. First, a sugar isolated by Anderson (15) from the water separated from the ether-alcohol extraction of lipoids from H-37, and designated A-8; second, an analogous sugar from the bovine strain; third, preparations from the Bacilli H-37, prepared by Heidelberger, Hbg 511 and 514; and fourth, similar preparations made by the H. K. Mulford Company, MB-200 (16). The guinea pigs used in these experiments were all tested with tuberculin (0.02 cc. O. T. Saranac) and were negative before the inoculation with tubercle bacilli. They all received 1,000,000 counted organisms (1/50 mg.) of a 10 day culture of human Strain H-37 obtained from Dr. S. A. Petroff. They were inoculated either intraperitoneally or subcutaneously in the groin, as shown in Tables II and III. The skin test was positive to O. T. in each instance before the injections of protein and polysaccharide were begun.

The rabbits were given 0.5 mg. of the Strain B-1 undissociated, intravenously from a 13 day culture. The experiments with the proteins and the polysaccharides were begun about 6 weeks after inoculation and were continued through 4 months. One (R 1344) of the 20 guinea pigs of the first series (inoculated Feb. 14, 1930), died 18 weeks after inoculation without having received any injection, and showed caseous inguinal lymph nodes and extensive tuberculosis of liver, spleen, omentum and lungs. Other animals that died following the injection of both protein and polysaccharide have shown marked lesions of the liver and spleen. In every case, with one exception, the routine was to take a rectal temperature first, then the specimens for the blood count and then give the injection. The exception was the first experiment on Chart 3, Guinea pig R 1323, in which the injection was given first. The amount of fluid injected was in each instance 1 cc. The polysaccharide and the water-soluble Protein 304 were dissolved in freshly distilled water. The alkali-soluble protein was dissolved in distilled water and then

TABLE II

Effects of Tuberculo-Proteins in Tuberculous Guinea Pigs

Dose	Animal number*	Date of infection with tubercle bacilli H_{37}	Type of protein injected	Date of injection of protein	Route of injection	Effect on temperature	Blood count before infection. White blood cells per c. mm.			Blood count before injection of protein. White blood cells per c. mm.			Maximum change in cells following protein. White blood cells per c. mm.			Result
							PMN	Ly	M	PMN	Ly	M	PMN	Ly	M	
mg.																
10	17 R 1329	2/14/30 (i.p.)	Mulford MA-100	4/11/30	i.v.	−10°	1970	5135	632	—	—	—	—	—	—	Death in $2\frac{3}{4}$ hours
5	9 R 1321	2/14/30 (i.p.)	"	4/10/30	i.p.	−7°	2651	2289	1325	6987	4658	2055	2744	817	480	Death in 16 hours
	40 R 1398	3/22/30 (i.p.)	"	5/ 5/30	i.p.	−3°	3036	1104	276	—	—	—	—	—	—	Death in $5\frac{1}{4}$ hours
	41 R 1399	3/22/30 (i.p.)	"	5/ 5/30	i.p.	±1°	—	—	—	—	—	—	—	—	—	Death within 24 hours
2.5	48 R 1406	3/22/30 (i.p.)	"	5/ 5/30	i.p.	−1.3°	—	—	—	—	—	—	—	—	—	Death within 24 hours
1	3 R 1315	2/14/30 (i.p.)	304	3/25/30	Intra-testicular	+1° −3°	1768	1224	340	3567	1537	1045	5905	305	152	Survival

	30 R 1342	2/14/30 (s.c.)	Alkali soluble protein (Johnson)	3/25/30	Intratesticular	+4.5°	5883	3330	1554	5200	9152	5616	19712	1888	507	Survival
	16 R 1328	2/14/30 (i.p.)	Mulford MA-100	4/16/30	i.p.	−1.8° +2.2° −3.9°	4140	5934	1380	2782	1575	735	8532	756	1404	"
0.1	3 R 1315	2/14/30 (i.p.)	"	4/21/30	i.p.	−.8° +3° −5°	1768	1224	340	2366	1820	364	3960	176	110	"
0.02	30 R 1342	2/14/30 (s.c.)	"	4/22/30	i.p.	−1.5° +3.4°	5883	3330	1554	12198	14766	2568	19276	2254	483	"
0.01	31 R 1343	2/14/30 (s.c.)	"	4/22/30	i.p.	+2.9°	6732	2856	408	5016	5700	684	11808	1065	1152	"
0.001	31 R 1343	2/14/30 (s.c.)	"	3/25/30	Intratesticular	+3.2°	6732	2856	408	12243	6237	4389	14700	2952	980	"
	36 R 1394	3/22/30 (s.c.)	"	4/23/30	i.p.	+2.5°	—	—	—	1527	1560	162	4972	526	396	"
	30 R 1342	2/14/30 (s.c.)	"	4/25/30	i.p.	−.7° +3.7°	5883	3330	1554	5475	9417	3285	16942	1576	985	"
	7 R 1319	2/14/30 (i.p.)	"	5/ 1/30	Intradermal	Negative −1.3° +1.4°	1260	714	126	4380	1440	180	7296	498	1462	"

* These are serial numbers of the work of the department covering a term of years.

TABLE II—*Concluded*

Dose	Animal number	Date of infection with tubercle bacilli H_{37}	Type of protein injected	Date of injection of protein	Route of injection	Effect on temperature	Blood count before infection. White blood cells per c. mm.			Blood count before injection of protein. White blood cells per c. mm.			Maximum change in cells following protein. White blood cells per c. mm.			Result
							PMN	Ly	M	PMN	Ly	M	PMN	Ly	M	
mg.			Milford													
0.0001	31 R 1343	2/14/30 (s.c.)	MA-100	4/25/30	i.p.	+2°	6732	2856	408	5202	4182	714	10033	1056	362	Survival
	3 R 1315	2/14/30 (i.p.)	"	6/ 4/30	i.p.	+1.8°	1768	1224	340	3605	721	515	4736	517	1175	"
	30 R 1342	2/14/30 (s.c.)	"	6 /4/30	i.p.	−.9° +1.7°	5883	3330	1554	9196	9196	1881	20349	4170	3876	"
0.00001	16 R 1328	2/14/30 (i.p.)	"	5/ 2/30	i.p.	Negative	4140	5934	1380	4212	819	702	7396	484	315	"
	11 R 1323	2/14/30 (i.p.)	"	6/11/30	i.p.	Negative	9891	4867	785	9398	7620	4318	23004	2840	1528	"
	20 R 1332	2/14/30 (i.p.)	"	6/10/30	i.p.	+2.0°	1026	3564	810	4089	2044	916	8925	447	0	"
	7 R 1319	2/14/30 (i.p.)	"	6/10/30	i.p.	+4.2°	1260	714	126	2208	621	621	2596	295	59	"

enough 1 per cent NaOH added to make the solution neutral to litmus. The Mulford Protein MA-100 was received in sterile salt solution, 10 mg. per cubic centimeter, and it was diluted with freshly made, sterile salt solution.

Killing Power of Tuberculo-Proteins and Polysaccharides in Tuberculous Guinea Pigs

As is shown in Table II, all five tuberculous guinea pigs which received from 10 to 2.5 mg. of the protein died within 24 hours; and all receiving less than 2.5 mg., seventeen injections in all, survived. Thus the minimal lethal dose for this protein lies between 2.5 and 1 mg.

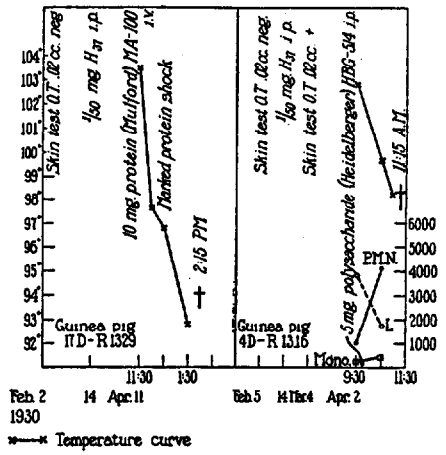

CHART 1

in tuberculous guinea pigs. A report of the killing power of protein prepared from the filtrate has been given by Seibert (17). In Chart 1 is shown the type of temperature curve following a lethal dose of the protein, for Guinea pig R 1329.

The injection was intravenous; the animal showed irregular and rapid breathing within 2 minutes of the injection. Subsequently there was involuntary defecation. The temperature fell precipitously 10°. The irregular breathing continued until death 4¼ hours later. The postmortem examination showed the omentum, spleen, and liver massively involved with tuberculosis; the lymph nodes were markedly caseous; the lungs had a moderate number of tubercles. There were numerous fresh hemorrhages especially marked around the tuberculous lesions in the omentum and in the lung. The lungs were markedly congested.

A more gradual fall in temperature, 7° in 7 hours, is shown in Chart 2, of Guinea pig R 1321, which received 5 mg. of the Protein MA-100 intraperitoneally.

The animal died in 16 hours after the injection. The postmortem examination showed extensive involvement of omentum, spleen, liver, and inguinal lymph nodes; there were a few tubercles in the lung. Fresh hemorrhages were not apparent at autopsy, but in sections there was marked congestion of the vessels of the lung and some hemorrhage into the tubercles. It will be noted in the chart that there was a fall in neutrophilic leucocytes, lymphocytes, and monocytes during the 7 hours that counts were made. All but one of the animals, R 1399, that died within 24 hours after the injection, had the marked fall in temperature.

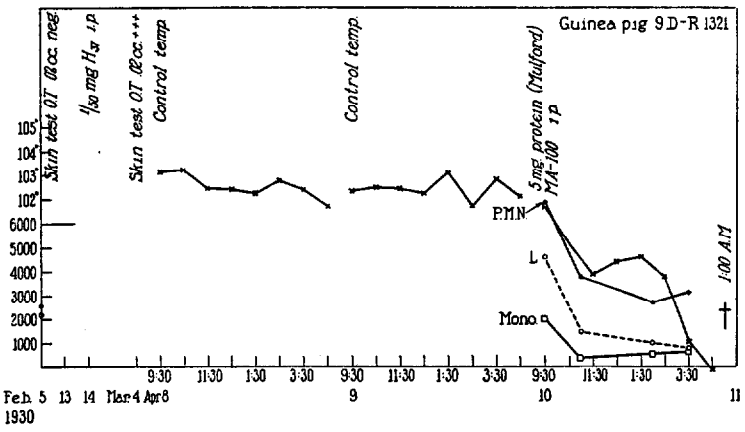

CHART 2

In this instance the changes in temperature were but slight; there was a drop of a degree and then a rise to the original level; the temperature had begun to fall when last taken and the animal died in the night. All of the animals that showed the fall in temperature were obviously quite sick, showing a marked toxic effect of the protein. The other animal that died after protein showed the same extensive involvement of liver, omentum, spleen, and lymph nodes. The lungs had a few tubercles and were markedly congested and in one case (R 1406) hemorrhagic.

The polysaccharides had also a certain killing power in sensitized guinea pigs, though it was by no means as consistently related to dosage as with the protein. In the original studies of Sabin, Doan, and Forkner (2, 3) six tuberculous guinea pigs received 10 mg. of the Polysaccharide A-8 intraperitoneally; two died within a few hours, two during the night after the experiment, while the others survived.

The polysaccharides in the present series did not show as marked a killing power for guinea pigs.

As shown in Table III, one guinea pig, R 1324, died in 4¼ hours after an intraperitoneal injection of 20 mg. of the A-8. The liver, spleen, omentum, and lymph nodes were only moderately involved; the lungs were markedly congested; but no hemorrhages were found in sections. This animal had survived two injections on the 2 preceding days of 10 mg. each of this polysaccharide. None of the animals receiving 10 mg. died; but R 1316 which had an intraperitoneal dose of 5 mg. of the Heidelberger polysaccharide died in 1¼ hours, the fall in temperature and the changes in the blood cells being shown in Chart 1. At postmortem there was extensive involvement of liver, omentum, and spleen. The lungs showed many tubercles, but were only moderately congested and had no hemorrhages. It is important to consider whether there was sufficient protein in this dose of the polysaccharide to account for the killing power and for the fall in temperature. The problem with the polysaccharide is complicated through not knowing the actual state of the nitrogenous contamination or its potency. Dr. Heidelberger found the nitrogen content of this sugar to be 0.34 per cent, so that if all of the nitrogen were calculated as protein, 1 mg. of polysaccharide would contain 0.021 mg. of protein; and 5 mg. would have only 0.105 mg. protein, which is below the minimal lethal dose of the protein whose potency we have been testing. On this basis it would take 100 mg. of the polysaccharide to contain 2.1 mg. of protein, 2.5 mg. having killed a guinea pig (Table II).

The question of a possible killing power of the polysaccharide here employed needs further study, with a larger series of experiments; but these experiments suggest that it either has a certain killing power in sensitized animals or it may enhance the killing power of a dose of protein too small to kill by itself. It should be stated that the manner of death is exactly like that of the lethal action of the protein.

In relation to the fall in temperature, on the other hand, it is interesting to note that the animal (Guinea pig R 1315, Table II) which received 0.1 mg. of the protein, the computed amount in the 5 mg. of polysaccharide, showed a final drop of 5° in temperature, so that if the fall in temperature in Guinea pig R 1316 (Table III) which succumbed to the polysaccharide was a direct effect of the substance injected rather than an indirect toxic effect on the animal, it might have been due to the protein introduced with the sugar.

It will be noted that one other animal which received 5 mg. of the sugar, R 1317, showed a fall in temperature and died 4 days later.

TABLE III

Effects of Tuberculo-Polysaccharides in Tuberculous Guinea Pigs

Dose	Animal number	Date of infection with tubercle bacilli H_{37}	Type of polysaccharide injected (with 1 cc. distilled H_2O)	Date of injection	Route of injection	Effect on temperature		Blood count before infection. White blood cells per c. mm.			Blood count before injection of polysaccharide. White blood cells per c. mm.			Maximum change in cells following polysaccharide. White blood cells per c. mm.			Result
								PMN	Ly	Mono	PMN	Ly	Mono	PMN	Ly	Mono	
mg.																	
20	12 R 1324	2/14/30 (i.p.)	A-8 original (Anderson)	3/13/30	i.p.	−3°		2618	2796	476	5400	750	1125	5525	378	252	Death in 4½ hours
	31 R 1343	2/14/30 (s.c.)	Bovine (Anderson)	4/17/30	i.p.		+4.1°	6732	2856	408	8008	5434	572	20976	456	0	Survival
10	15 R 1327	2/14/30 (i.p.)	A-8 purified (Anderson)	3/12/30	i.p.		+2.7°	5376	7056	2352	12954	4000	2095	5760	976	525	"
	12 R 1324	2/14/30 (i.p.)	A-8 purified (Anderson)	3/11/30	i.p.	−0.8°	+3.2°	2618	2796	476	5226	2821	1202	6732	624	288	"
	12 R 1324	2/14/30 (i.p.)	A-8 original (Anderson)	3/12/30	i.p.	−2.0°	+2.8°	2618	2796	476	4416	1449	966	28000	412	8800	"
	15 R 1327	2/14/30 (i.p.)	Heidelberger Hbg 514	3/21/30	i.p.	−1.2°	+1.4°	5376	7056	2352	2300	5635	3220	6240	855	380	"
	15 R 1327	2/14/30 (i.p.)	Bovine crude II (Anderson)	3/13/30	i.p.	Negative	+0.6°	5376	7056	2352	14418	1424	1246	2860	553	220	
	18 R 1330	2/14/30 (i.p.)	Bovine crude I (Anderson)	3/13/30	i.p.		+2.7°	2160	1480	240	5865	1870	595	8733	817	30	"
5	11 R 1323	2/14/30 (i.p.)	Mulford MB-200	4/ 8/30	i.v.	−0.9°	+4.2°	9891	4876	785	No counts			No counts			"

														Heart's blood			
5	4 R 1316	2/14/30 (i.p.)	Heidelberger Hbg 514	4/ 2/30	i.p.	−4.5°		4160	1728	448	1080	3888	216	15164	6244	669	Death in 1¾ hours
	7 R 1319	2/14/30 (i.p.)	Mulford MB-200	4/ 7/30	i.p.		+4.5°	1260	714	126	2982	1008	126	5772	232	1170	Survival
	5 R 1317	2/14/30 (i.p.)	Mulford MB-200	3/31/30	i.p.	−3.7°		2838	2709	516	5775	1750	1225	9625	516	1625	Died 4 days later
2.5	6 R 1318	2/14/30 (i.p.)	Heidelberger Hbg 514	4/ 4/30	i.p.		+2.1°	3168	1485	198	3136	1617	98	7830	130	720	Survival
1.0	10 R 1322	2/14/30 (i.p.)	Mulford MB-200	4/14/30	i.p.	−4.8°	+4.7°	5856	4309	773	8532	5372	1738	26080	1810	530	"
	7 R 1319	2/14/30 (i.p.)	Heidelberger Hbg 514	4/28/30	i.p.	With secondary rise 4/29	+1.8°	1260	714	126	3321	1968	738	4009	594	346	"
0.1	19 R 1331	2/14/30 (i.p.)	Heidelberger Hbg 514	5/22/30	i.p.		+2.3°	1971	1022	584	4674	5535	615	9130	2300	1660	"
0.01	11 R 1323	2/14/30 (i.p.)	Heidelberger Hbg 514	5/29/30	i.p.	Negative		9891	4867	785	13296	9418	2770	9747	2565	3591	"
	20 R 1332	2/14/30 (i.p.)	Heidelberger Hbg 514	5/23/30	i.p.		+2.3°	1026	3564	810	5960	7003	1937	7802	1034	470	"
	7 R 1319	2/14/30 (i.p.)	Heidelberger Hbg 514	6/ 3/20	i.p.	−1°	+1°	1260	714	126	3752	728	1064	8034	824	1960	"

At the postmortem examination, extreme involvement of liver, spleen, omentum, and peripheral lymph nodes was found together with many tubercles in the lungs. There were extensive hemorrhages. They involved many of the lymph nodes and some of the organs, as well as the vessels in the skin and in the subserosal layers of the peritoneal lining. It may be that a sustained fall in temperature after the injection of either protein or polysaccharide indicates, even if the animal survives 24 hours, that it is not likely to survive the disease for many days.

In general the animals which died after both protein and polysaccharide had extensive involvement of the liver, spleen, omentum, and lymph nodes, and a moderate number of tubercles in the lungs. They all showed congestion of the vessels of the lung but no edema; most of them had hemorrhages around the tubercles in the lung and around the caseous areas in the omentum. The lymph nodes were congested.

Reaction to Sublethal Doses of Protein and Polysaccharide in Tuberculous Guinea Pigs

The animals which survived the injection of either of these substances did not show any marked effects except on the temperature and on the blood cells. They were not clinically ill.

The results were the same for both substances regardless of the route of injection, whether intravenous, intraperitoneal, or intratesticular. The type of the changes in the blood cells and the temperature are shown in Tables II and III; but the type of reaction is better indicated by curves as shown in Charts 3 to 5. A typical temperature reaction involved a preliminary fall lasting from ½ to 2 hours, followed by a gradual rise of from 2 to 4.5° with a return to the original level in approximately 5 hours. The positive reaction was not only a change in temperature but a specific type of curve. The fall occurred at once after the injection; it was, however, sometimes much shortened or even suppressed, but the subsequent rise and return to the original level were constant. The sublethal reaction for the polysaccharide is shown by the first curve in Chart 3, R 1323, the animal having received 5 mg. of the Mulford polysaccharide intravenously. The injection was made before a preliminary temperature had been taken and no blood counts were taken. The animal had a slight chill but no other symptoms. Similar curves for the different proteins are shown in Chart 4, R 1342. The animal received an injection of 1 mg. of the alkali-soluble protein (Johnson) intratesticularly 5 weeks after inoculation. It was highly sensitized; the temperature fell slightly for an hour and then rose 4.5° by five o'clock. It was still above 103° the next morning, the original level having been 102°. It will be noted that the leucocytes rose in this instance while both lymphocytes and monocytes fell.

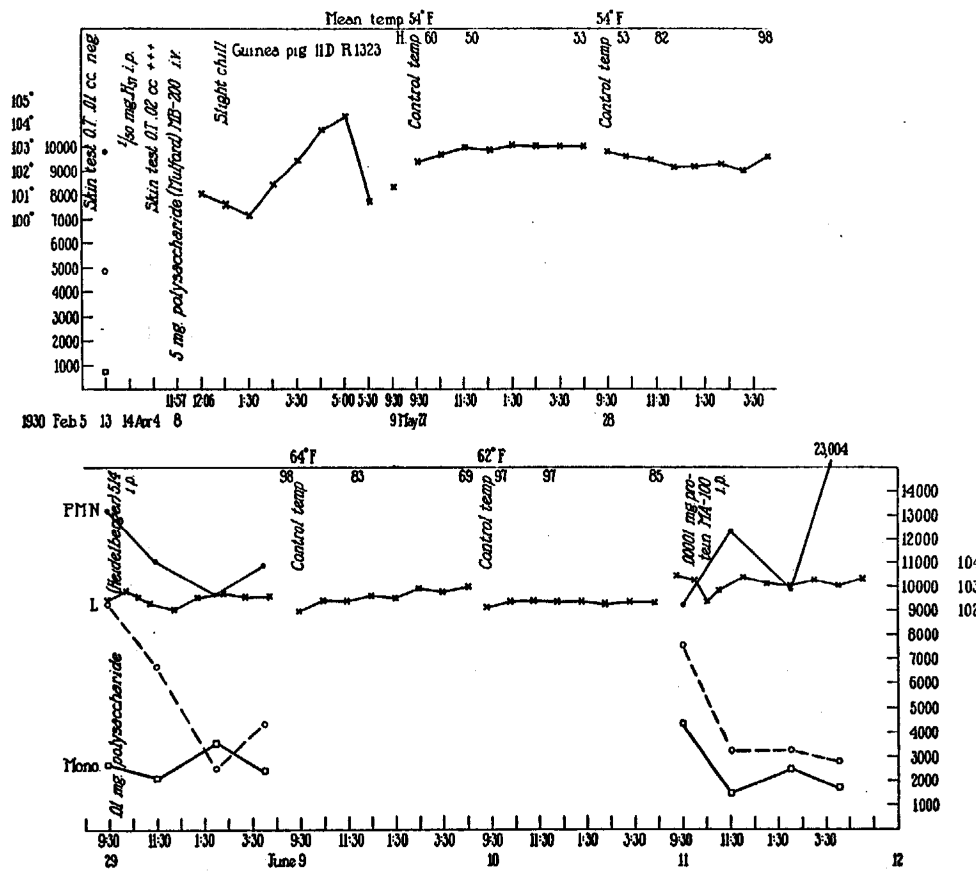
Mean temp 54°F
54°F
Guinea pig IID R1323
Skin test O.T. .01 cc. neg
1/50 mg. H_{37} i.p.
Skin test O.T. .02 cc +++
5 mg. polysaccharide (Mulford) MB-200 i.v.
Slight chill
Control temp
1930 Feb 5 13 14 Apr 4 8
9 May 27
28
64°F
62°F
23,004
PMN
L
Mono.
(Heidelberger) 5.14 i.p.
.01 mg. polysaccharide
.00001 mg. protein MA-100 i.p.
June 9
10
11
12

CHART 3

Biological Titration of the Tuberculo-Protein and Polysaccharide in Tuberculous Guinea Pigs.—Since the reaction of the temperature and of the blood cells to these two substances was the same and since each was contaminated with the other, it was necessary to see if one substance could give the reaction in such small doses as to exclude the other. The experiment with titrations of these two substances is shown in Tables II and III.

All but one of the guinea pigs receiving injections of the protein of from 1 to 0.0001 mg. gave the characteristic temperature curve and fall in lymphocytes. The one negative reaction was in Guinea pig R 1319, Chart 5, following the injection of 0.001 mg. of the Mulford protein given by the intradermal route. With the next dilution, containing 0.00001 mg. of protein, the reaction became varied; two were negative, one moderately and one markedly positive. This dosage thus approaches the limits of the reaction to the protein.

On the other hand, in Table III it is clear that the inconstant temperature reaction was obtained with the dose of 0.01 mg. of the polysaccharide; one animal was negative, a second showed a slight reaction and the third had a rise of 2.3°. On the basis that 1 mg. of the Heidelberger polysaccharide contains 0.021 mg. of protein or protein degradation products, 0.01 mg. would contain 0.00021 mg. of protein, which is twice the dose of protein which gave a consistently positive reaction.

There was probably enough protein, or its degradation products, to account for the temperature reaction in all of the experiments with the polysaccharides, provided the protein was in an active form.

Variations in Temperature Induced in Tuberculous Guinea Pigs by Protein and Polysaccharide

Certain interesting points can be made out in this connection.

In Chart 3 it will be noted that the guinea pig was highly sensitive to polysaccharide (protein) as shown by the first temperature reaction after inoculation with tubercle bacilli. During the months of May and June the control temperature curves were constant. These control periods are an important part of the experiment for they show that there is for the most part relatively little fluctuation in temperature from the disease itself. In only one animal of the entire series was there an afternoon temperature during one of these control periods; in this animal, R 1332, on one occasion the temperature was steady for the morning hours but rose suddenly 3.2° between one-thirty and two-thirty and then fell gradually to the original level. The cause of this rise was not determined. In Chart 3 it will be seen that the temperature remained normal both after 0.01 mg. of the Heidel-

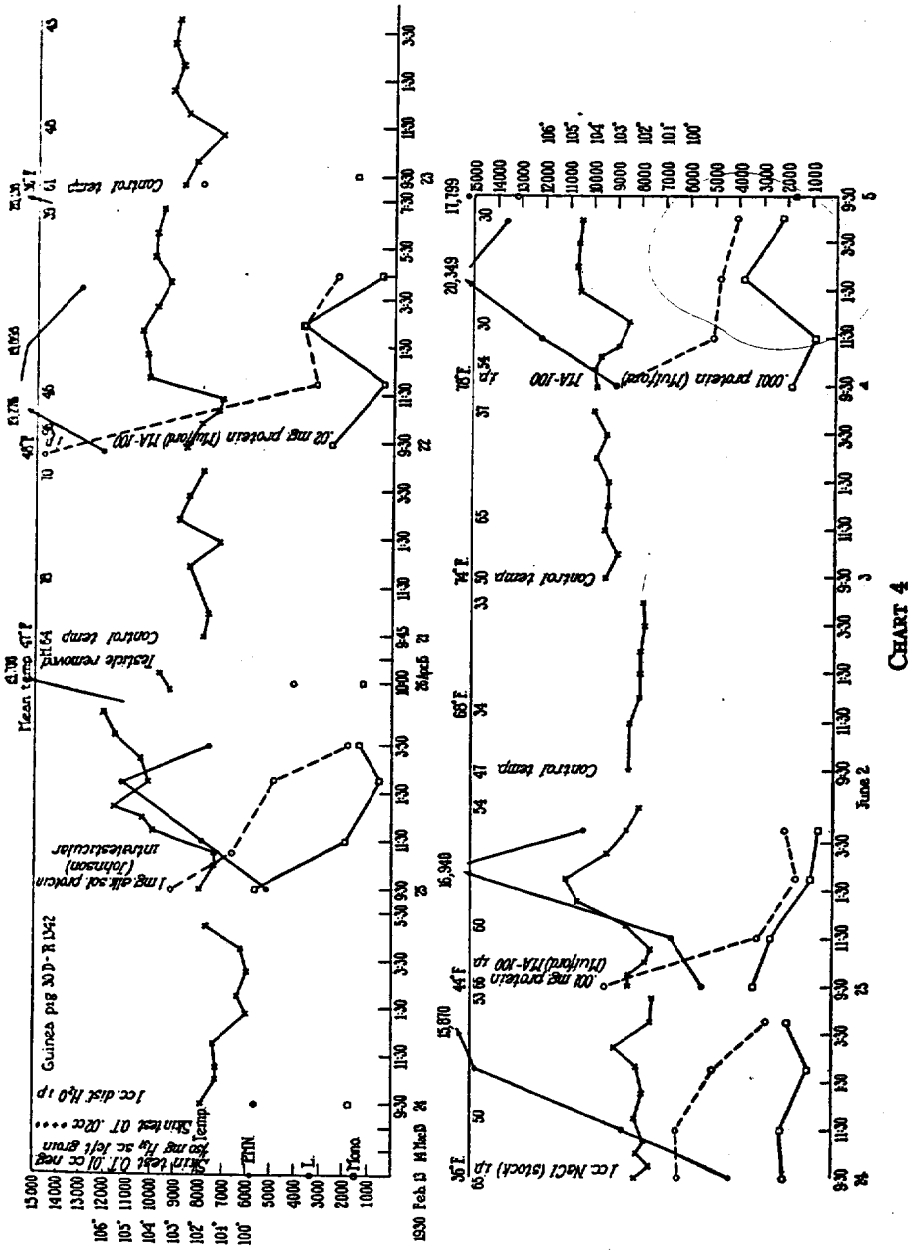

CHART 4

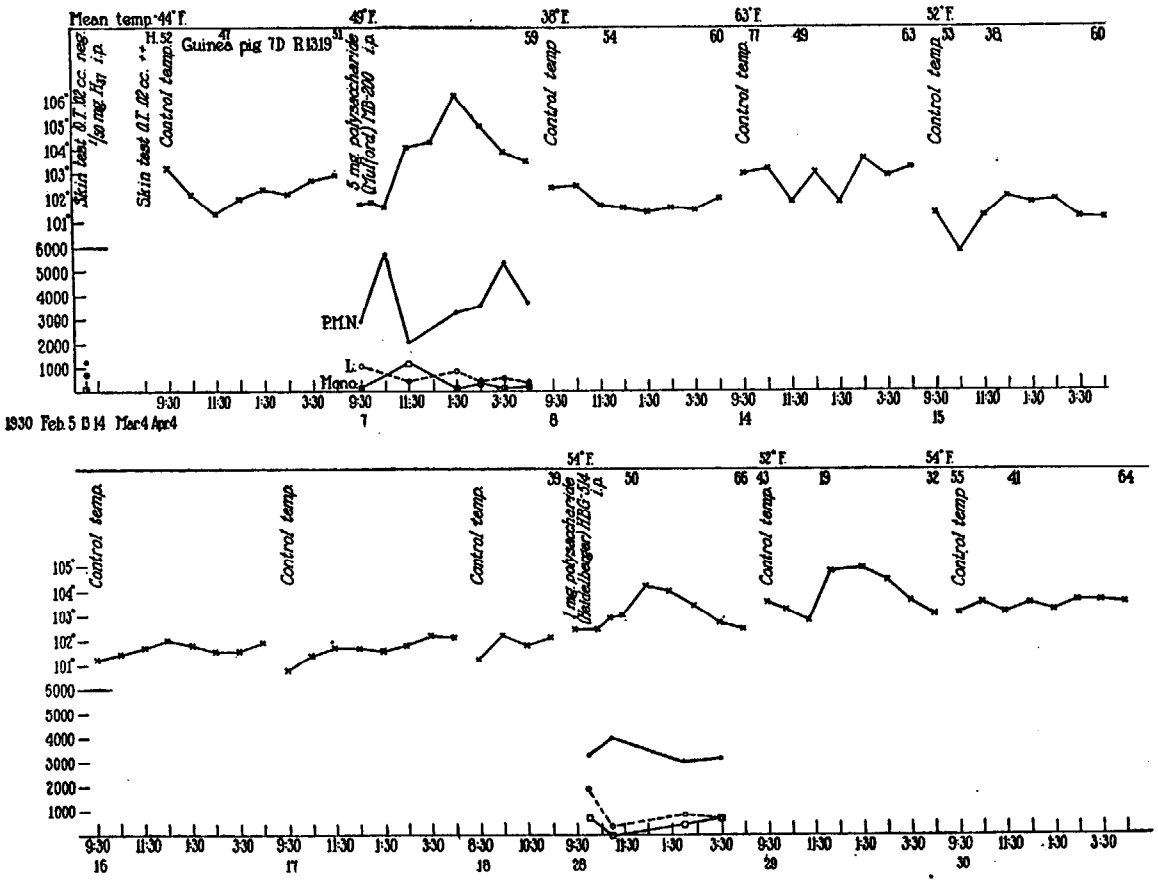

CHART 5—*Continued on Next Page*

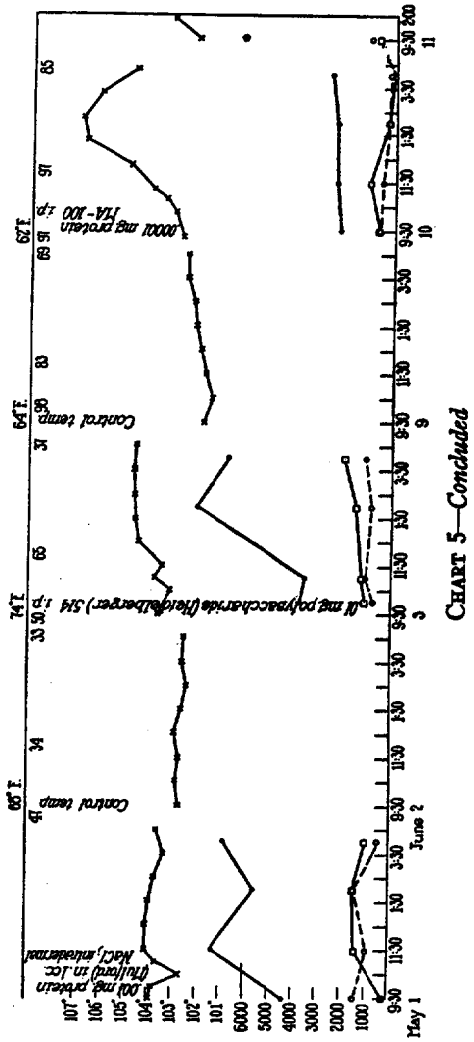

CHART 5—*Concluded*

berger polysaccharide and after 0.00001 mg. of the Mulford protein; but on both of these days, the lymphocytes fell markedly and the neutrophilic leucocytes were irregular. Thus the reactions on the blood cells are not to be correlated with the change in temperature.

Chart 4 represents a long experiment with Guinea pig R 1342. The first experiment shows that there was no rise in temperature, but rather a fall after an injection of 1 cc. distilled water intraperitoneally. The water was freshly distilled from glass, boiled and used as soon as sufficiently cooled. A second control with distilled water in another guinea pig, R 1332, showed a steady reaction. The intratesticular route of injection was employed in R 1342 to study the tissue reaction as reported by Long (18). 21 days later the testis was removed and showed the marked degeneration described by Long.

By the twenty-first of April the control temperature was moderately steady, and the following day the animal gave a positive reaction to 0.02 mg. of the Mulford protein. The rise in leucocytes and the fall in lymphocytes were extreme; it is interesting that the lymphocytes had returned to 8000 cells per cubic millimeter by the next morning, though not to the original level. On the twenty-fourth of April a test was made with salt solution which had been sterilized and sealed several days previously; there was a slight rise in temperature in the afternoon which did not occur with freshly made salt solution. The rest of the experiment had to do with the minute doses of the protein, 0.001 and 0.0001 mg., with less reaction in temperature to the smaller dose.

In the intervening days, it will be noted that the level of the temperature of the guinea pig was a degree higher on the third of June than on the preceding day. This phenomenon has occurred both with normal and tuberculous guinea pigs. The mean temperatures for New York City, together with three readings for humidity, reported at 8 a.m., at 12 noon, and at 8 p.m., by the Weather Bureau, United States Department of Agriculture, are included in the charts. In Chart 4 it will be noted that with a rise of 6°F. between June 2 and 3, the temperature of the guinea pig was a degree higher on the second day. Comparing the humidities on June 2 and 3 it will be noted that on June 2 there was a fall of 13 points between 8 a.m. and noon, and on the next day a rise of about the same amount, namely 15 points, so that the change in temperature of the animal in this instance has run parallel with the change in atmospheric temperature rather than with the variation in humidity.

Chart 5 represents another long series of experiments. This guinea pig, R 1319, was markedly sensitive to an injection of 5 mg. of the Mulford polysaccharide on April 7, about 7 weeks after infection. The original control temperature was somewhat irregular, as frequently happens the first time temperatures are taken. The change in humidity was not great. It will be noted that the temperature of the guinea pig was entirely steady on the day after the injection, but on the 14th and 15th of April was irregular and ran nearly 2° lower on the 15th. There was a drop in atmospheric temperature of 11°F. The temperature of the animal became

quite level again and on the 28th of April, it showed an average temperature reaction to 1 mg. of the Heidelberger polysaccharide. The next day there was a secondary rise, practically a duplicate of the curve of the preceding day with no change in the weather, and for 3 days the temperature of the animal was at a

TABLE IV

Effects of Tuberculo-Protein and Polysaccharide on Normal Guinea Pigs

Dose	Number of animal	Date of injection	Temperature after protein Ma-100	Fall of lympho-cytes	Date of injection	Temperature after polysaccharide (Heidelberger)		Fall of lympho-cytes
mg.				*per cent*				*per cent*
20	R 1433	6/ 6/30	+1.3°					
15	R 1430				4/14/30	−2.5°	+3.5°	70
10	R 1432	6/ 6/30	+1.9°					
	R 1425				4/10/30		+1.7°	87
5	R 1425				4/ 8/30	−1°	+1.5°	—
1	R 1430				4/17/30	−2°	+3.4°	50
0.1	R 1430				5/22/30	−1.2°	+1.2°	77
0.01	R 1430	4/30/30	+1.5°	70				
	R 1432				5/28/30	−2°	+2.5°	61
	R 1433				5/29/30		+1°	65
	R 1436				6/ 3/30	−5°	+1.6°	77
	R 1425				5/23/30	−1.3°	+0.7°	69
0.001	R 1435	5/ 1/30	+1.6°	66				
	R 1432	5/ 2/30	+2.4°	63				
0.0001	R 1433	5/ 2/30	Negative	48				
	R 1436	5/ 1/30	−1.5° +1°	32				
0.00001	R 1434	5/ 2/30	Negative	76				

higher level than before the injection. We have seen no other secondary rise of this magnitude, but in three instances it has taken 3 days for the temperature to come down to the previous level (R 1323, Chart 3, after 5 mg. of Mulford polysaccharide; R 1331, (19) after 0.1 mg. of the Heidelberger polysaccharide; and Rabbit R 1365, Chart 8, after the Heidelberger polysaccharide). On May 1, R 1319, Chart 5, there was a negative reaction to an injection of 0.001 mg. of protein MA-100 given intradermally. On the third of June there was a slight

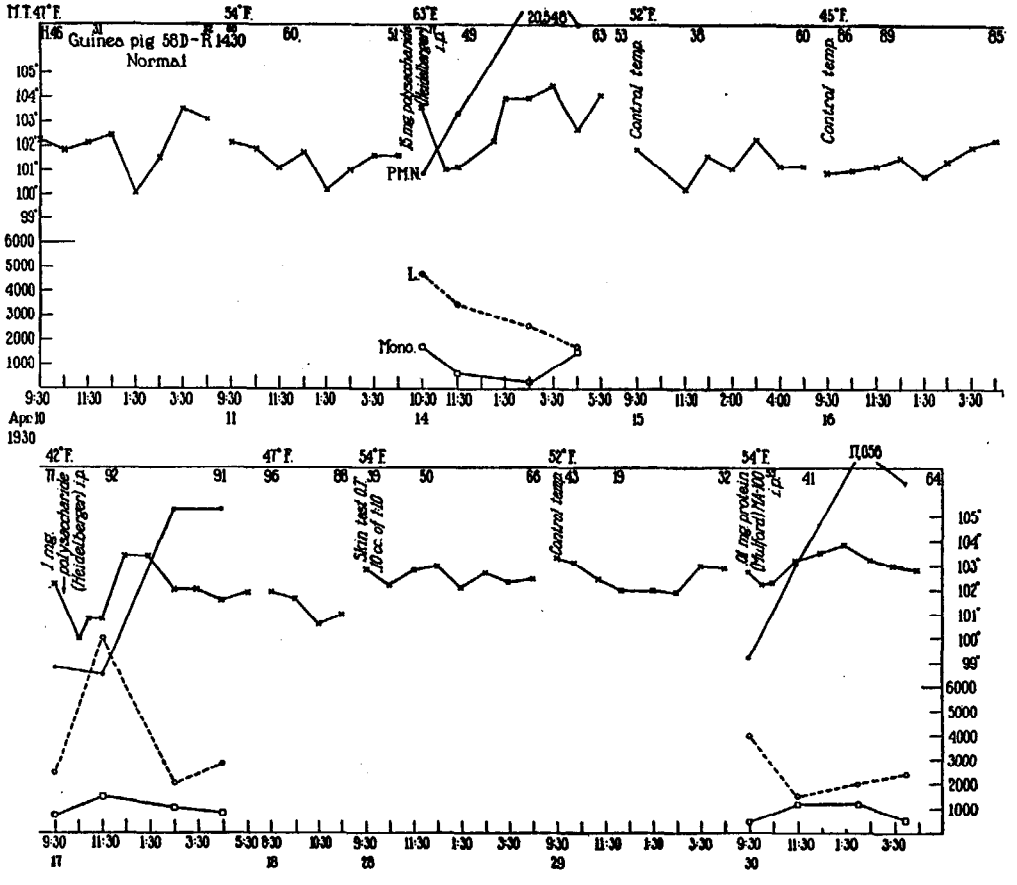
Guinea pig 58D-R1430
Normal
PMN
L.
Mono.
Control temp.
Control temp.
Skin test 0.7 c.c. of 1/10
Apr. 10
1930

CHART 6

reaction to 0.01 mg. of the Heidelberger polysaccharide, but 7 days later the animal proved to be highly sensitive to the Mulford protein in a dilution of 10^{-8}. It will be noted also that the temperature of the guinea pig during the control period of June 9, was lower by 2°C. than the initial temperature of June 3. Between these 2 days the environment differed by 10°F. This guinea pig showed low lymphocytes throughout the experiment but they fell with every injection except after the 0.01 mg. of the polysaccharide.

Effects of Tuberculo-Protein and Polysaccharides on the Temperature of Normal Guinea Pigs

The results of experiments with two types of fractions are shown in Table IV and Chart 6.

For all of the work the Mulford Protein MA-100 and the Heidelberger Polysaccharide 514 were used. It will be seen that with the normal animal the rise of temperature was more moderate throughout than with the sensitized animal; the type of curve was, however, the same. It is likewise clear that the biological titres of polysaccharide and protein exhibit the same relationship in the normal guinea pig as in the sensitized; however the necessary amounts for normal animals are 10 to 100 times greater. For the polysaccharide, the reaction was either negative or slight with doses of 0.1 and 0.01 mg.; while with the protein the limit of reaction was 0.001 mg. and the dose of 0.0001 mg. was negative. In Chart 6 it will be noted that during the control periods the temperatures were more irregular but were not at a lower level than with the tuberculous animals. There were more of these irregular control temperatures in normal than in tuberculous guinea pigs.

Effects of Tuberculo-Proteins and Polysaccharides on Tuberculous and Normal Rabbits

Fewer experiments were made with rabbits than with guinea pigs. With two tuberculous rabbits, it was determined that the protein from the Strain H-37 gave a rise in temperature in small doses. Rabbit R 1366 was given 1 mg. of the Protein MA-100 10 weeks after inoculation with 0.5 mg. bovine Strain B-1; the temperature fell 0.5° and then rose 2°; 2 days later the reaction to 0.001 mg. of the same protein was similar but with a rise of only 1.2°. Rabbit R 1367, inoculated with tuberculosis at the same time as R 1366, was given 0.01 mg. of the same protein in 10 weeks and showed a fall of 1.2° and a subsequent rise of 2.1°; while 2 days later the rise in temperature after the injection of 0.0001 mg. was of the same magnitude, 2°. After all four injections in these two animals, the leucocytes rose and the lymphocytes and monocytes fell. Considering the difference in size of rabbit and guinea pig, the tuberculous rabbit inoculated with the bovine strain of organisms proved to be quite sensitive to the protein from the human strain of tubercle bacillus.

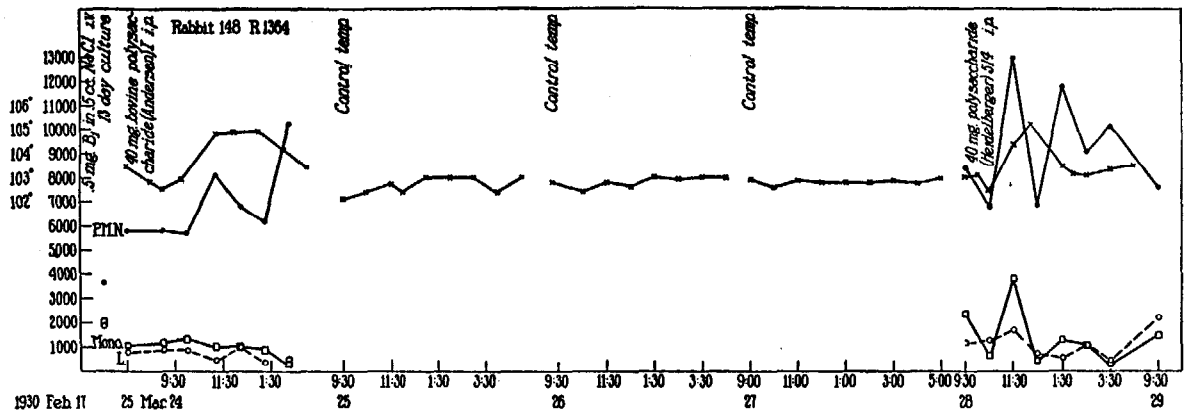

CHART 7

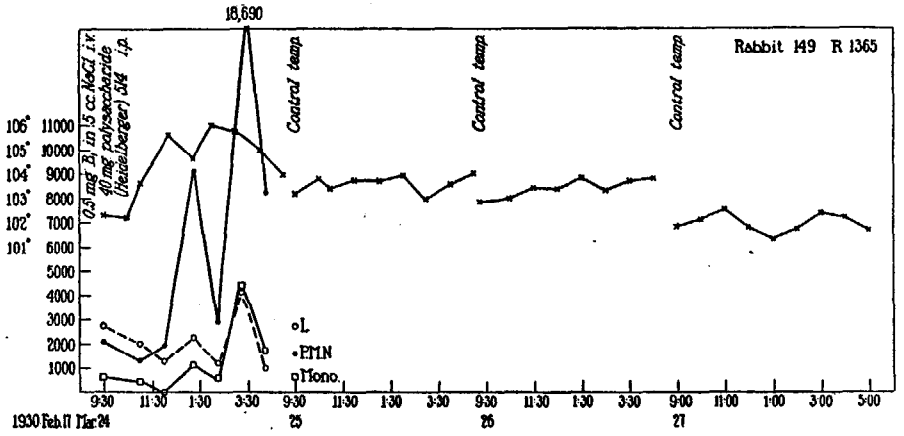

CHART 8

In two other tuberculous rabbits, R 1364 and R 1362, a comparison was made between the reaction of a polysaccharide isolated by Anderson from the bovine organism, analogous to the A-8 from the human strain and the polysaccharide isolated by Heidelberger from the human strain. In both instances the reaction was practically identical and is shown in Chart 7 for R 1364. A more marked rise in temperature following the Heidelberger polysaccharide was shown by another rabbit, R 1365, Chart 8. The chart illustrates very well the delayed return of the temperature to the original already noted.

Controls

The polysaccharides, the Protein 304, and the alkali-soluble protein were all injected into the guinea pig in 1 cc. water freshly distilled from glass. In the rabbits 2 cc. of fluid were used. The Protein MA-100 was received from the H. K. Mulford Company in sealed ampules in sterile salt solution. It was thus necessary to study the effects of these diluents. The freshly distilled water or salt was boiled and used as soon as sufficiently cooled. Neither the water nor the freshly made salt solution gave any rise in temperature. The curves of temperature for distilled water and for a stock salt solution are shown in Chart 4, and the records are given in Table V. In general both the distilled water and the salt solution did not give a positive curve in temperature. The distilled water was either negative or showed a slight fall as on Chart 4. The stock salt solution, which had been kept in sterile flasks gave a slightly more irregular curve, Chart 4, than the freshly prepared solution.

In these experiments it has been found that control temperatures at hourly intervals should be taken for 2 or 3 days preceding each experiment and after each experiment until the temperature reaches the original level. This may take 4 or 5 days.

Concerning these control temperature reactions, two factors must be considered; first, the level at which the temperature runs, and second, the steadiness of the reaction during the day. Our charts show differences in level of as much as 2°. This difference in the animal is probably related to differences in atmospheric temperature, which, of course, could only be tested when the animals were not in artificially heated rooms. The irregular temperature reactions are more difficult to evaluate because more factors may be involved. It is probable that variations in humidity can affect the temperature of the animal, but abnormal conditions, such as accidental infections must also be taken into consideration.

TABLE V

Effects of Distilled Water and Salt Solution in Tuberculous Guinea Pigs

Animal number	Date of infection with tubercle bacilli H_{37} 1,000,000 organisms	Type of fluid injected (1 cc.)	Date of injection of fluid	Route of injection	Effect on temperature	Blood count before infection. White blood cells per c. mm.			Blood count before injection of fluid. White blood cells per c. mm.			Maximum change in cells following injection of fluid. White blood cells per c. mm.			Result
						PMN	Ly	Mono	PMN	Ly	Mono	PMN	Ly	Mono	
30 R 1342	2/13/30	Distilled H_2O	3/24/30	i.p.	Irregular −1.9° +1.7°	5883	3330	1554	10089	5564	1770	—	—	—	Survival
14 R 1326	2/13/30	Distilled H_2O	6/ 5/30	i.p.	Negative +0.8°	9638	13806	2084	11712	4224	2880	14980	2041	1365	"
20 R 1332	2/13/30	Distilled H_2O	6/ 5/30	i.p.	Negative −0.7° +0.4°	1026	3564	810	5746	1774	929	7169	1422	1148	"
30 R 1342	2/13/30	Stock salt solution	4/24/30	i.p.	Irregular ±1.0°	5883	3330	1554	4260	6390	1988	15870	2760	1525	"
31 R 1343	2/13/30	Fresh salt solution	4/24/30	i.p.	Negative	6732	2856	408	6840	3720	1320	11160	1878	552	"
57 R 1425	Normal	Distilled H_2O	6/ 6/30	i.p.	Negative	3504	1679	1971	—	—	—	—	—	—	"

Effects of the Injection of the Tuberculo-Proteins and Polysaccharides on the Blood Cells

In this series of experiments only immediate effects of the injection have been considered. The blood counts for tuberculous guinea pigs after protein and polysaccharide are shown in Tables II and III.

The total numbers of neutrophilic leucocytes, lymphocytes, and monocytes are given before the infection with tubercle bacilli, in the first column; in the second column are the corresponding figures for the count taken just before the injection of either protein or sugar; while in the third are recorded the greatest change in these three strains. This third column therefore does not represent a single count as was true for the first two columns. After the injection of the protein there was a rise in leucocytes in every guinea pig except the second animal in Table II, R 1321, which died in 16 hours; this was the only one of the fatal cases in which counts were made. In every animal there was a fall in lymphocytes, which for the most part showed little or no tendency to recover; the monocytes have also fallen in every instance but have shown a tendency toward a subsequent rise before the end of the 7 hours of the experiment. The average fall in lymphocytes in tuberculous guinea pigs after the protein, computing from the percentages rather than from the total numbers, was 63 per cent.

After the injection of the polysaccharides the total numbers of lymphocytes fell in every instance but one; this case was the last experiment in Table III, R 1319, shown in Chart 5, after the injection of 0.01 mg. of the Heidelberger polysaccharide, in which the lymphocytes were very low before the injection. In this animal, the total numbers of lymphocytes rose slightly but the percentages fell. In two instances, the percentage of lymphocytes rose while the total numbers fell; (R 1324 after A-8 on 3/13/30, and R 1327 after the bovine polysaccharide on 3/13/30). Including these percentages in the average, the lymphocytes fell 45 per cent after the polysaccharides, as is shown in Table III. Six normal guinea pigs showed a fall in lymphocytes of 55 per cent in 7 hours after tuberculo-protein, and eight showed a fall of 65 per cent after tuberculo-polysaccharide.

Non-Specific Protein Fever

It has long been known that the injection of proteins causes fever and a leucocytosis.

In 1890 Buchner (19) showed that bacterial proteins injected subcutaneously gave a local reaction of aseptic pus. The mechanism of this phenomenon was then studied by Roemer (20, 21) who found that an intravenous injection of bacterial protein gave a leucocytosis which was maximum in 8 hours. Goldscheider and Jacobs (22) then found that leucopenia preceded the leucocytosis using an extensive series of stimuli, organ extracts, bacterial proteins as well as

proteins from other sources. Arneth (23) then demonstrated that 8 hours after the injection of peptone intravenously in rabbits, at the height of the leucocytosis, there were 3 per cent myelocytes in the blood stream, showing a replacement of leucocytes from the bone marrow. In 1923 Hussey (24) discovered that the same phenomenon occurred after the injection of a considerable series of salts, sodium chloride, sodium carbonate, potassium phosphate, lithium nitrate, and sodium sulfate, and stressed the fall in mononuclear cells, finding that it was about 70 per cent in 3 hours. More recently Beard and Beard (25) have followed the blood cells in eight rabbits every 10 minutes for 5 hours after the intravenous injection of from 10 to 15 cc. of salt solution varying from 1 to 2.5 per cent. By making the counts at such frequent intervals they demonstrated that all three groups of the white cells, leucocytes, lymphocytes, and monocytes, fall immediately after the injection and that the maximum leucopenia is reached in about an hour. At this time the neutrophilic leucocytes start to rise, while the lymphocytes and monocytes continue to fall. The monocytes then begin to rise but in their experiments did not reach their original level in 5 hours, while the lymphocytes were lower by approximately 2000 cells at the end of the experiment.

This type of reaction is well shown for the tuberculous guinea pig, Chart 4, R 1342. By taking the count 2 hours after injection, the fall in the leucocytes was missed but the subsequent rise is clear. It will be noted that before each injection shown in this chart, the lymphocytes were high and showed a marked fall with the lowest level, with one exception, at the end of 7 hours. They had recovered in varying degrees by the next morning, but only in one instance did they exceed the level of the time before the injection. The monocytes varied somewhat in reaction in these tuberculous guinea pigs, but as shown in Chart 4, after a fall during the first 2 hours, they showed a tendency toward a temporary recovery in 5 hours with subsequent fall at the seventh hour.

As is shown in Table V the effect of the injection of distilled water on the blood cells may be slight as in Guinea pig R 1326, or negative as in R 1332; while the injection of salt solution is followed by the characteristic changes already described.

It is clear that the injection of proteins, salts and sugars has constant effects on the blood cells at quite specific time intervals. There is an immediate leucopenia followed by a leucocytosis, in which the three strains of circulating white cells fall at different rates and return at different times. The leucocytes start to return first; then the monocytes, and finally the lymphocytes. This reaction is elicited in the tuberculous animal by all the proteins tested; it also follows the injection of polysaccharide in concentrations containing an amount of nitrogen too small to cause a temperature reaction. The rise

in leucocytes in tuberculous animals is not consistently greater than is recorded in the literature, though several counts of over 20,000 pseudo-eosinophilic leucocytes are shown in Tables II and III. The fall in lymphocytes of 65 per cent corresponds with the 70 per cent for all mononuclear cells recorded by Hussey. The question suggests itself of whether the tuberculo-protein and the polysaccharides may not affect the proportion of monocytes to lymphocytes and so be concerned with the cellular factors of resistance to infection.

SUMMARY

The temperature reaction in tuberculous and normal guinea pigs and rabbits is elicited by the tuberculo-protein and probably not at all by the polysaccharides. The polysaccharides may have some killing power under certain conditions, but this is not as consistently related to dosage as in the case of the proteins. Both proteins and polysaccharides cause a change in the white blood cells when introduced by any route.

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