The Journal of Immunology

RESEARCH ARTICLE | JULY 01 1938

Phospholipids and Cholesterol in Plasma of Immunized Horses FREE

Augustus Wadsworth; ... et. al

J Immunol (1938) 35 (1): 55–69.
https://doi.org/10.4049/jimmunol.35.1.55

PHOSPHOLIPIDS AND CHOLESTEROL IN PLASMA OF IMMUNIZED HORSES

AUGUSTUS WADSWORTH AND L. W. HYMAN

From the Division of Laboratories and Research, New York State Department of Health, Albany

Received for publication February 25, 1938

A previous paper (1) records the distribution of the lipids and the degenerative lesions in the livers of nonimmunized horses and of those under immunization for the production of therapeutic sera. This report includes a study of the lipid constitution of the plasma and of the character of the variation in phospholipid and cholesterol content before and at intervals during the period of immunization with diphtheric and tetanal toxins, streptococcal toxin and culture, and meningococcal and pneumococcal cultures.

The investigations of Marie (2–4) on the changes in cholesterol have been followed by other studies of the lipids by Danysz-Michel and Laskownicki (5), Damboviceanu and Ionesco-Mihaiesti (6, 7), as well as Felton and Kauffmann (8), but the experimental conditions differ so that the results are not altogether comparable with those obtained in these studies. Also, some of the methods of analysis differ materially from those used in this and other studies (9).

METHODS OF IMMUNIZATION

Diphtheric toxin which was prepared with a special strain (no. 3203) previously described (10) was injected at intervals subcutaneously in gradually increased dosage (11). Three horses (nos. 380, 421, and 463) died of an acute toxemia following the first or second dose; the serum of each, however, had less than 0.002 unit of antitoxin per cubic centimeter before inoculation.

Tetanal toxin—later toxin and alum—were likewise injected

subcutaneously at intervals, in increasing doses. All of the horses had received two doses of tetanal toxoid and alum about six months before active immunization was begun.

Standard procedures (12) for the production of therapeutic sera were used in the remaining instances, as follows:

Living streptococci were injected into a mass of solidified agar under the skin of the horse. Later, the doses of living streptococci were supplemented by toxin injected subcutaneously at regular intervals.

Suspensions of living meningococci in physiological salt solution were given intravenously in gradually increased dosage.

Killed pneumococci in suspension, followed by living pneumococci, either whole culture or suspension, were injected intravenously in gradually increased doses.

METHODS OF ANALYSIS

The horses were not fed for 15 hours previous to bleeding. The specimens for plasma-analyses contained 0.1 per cent of potassium oxalate added as powder. Extractions were made according to the method of Bloor (13) with a mixture of 95-per-cent alcohol and ether (3:1), both redistilled. Thirty-seven and one-half cubic centimeters of plasma were extracted and the mixture was made up to 500 cc.

Phospholipids

Phosphorus was determined in the alcohol-ether extract and calculated as lecithin. Thirty cubic centimeters of the alcohol-ether extract were treated with 1.25 cc. of magnesium nitrate (saturated solution), evaporated to dryness in a 50-cc. beaker, ignited over a flame, treated with 0.7 cc. of concentrated nitric acid and a few cubic centimeters of water, and heated. After filtration, 2 cc. of nitric acid containing sulfuric acid were added, the solution was made up to a 15-cc. volume, and the determination completed according to the method of Pregl (14).

Cholesterol

Total and free cholesterol were determined according to the gravimetric, digitonin methods described by Osato and Heki

(15). For the determination of total cholesterol, 25 cc. of alcohol-ether extract of plasma were used; for the free-cholesterol determination, 75 cc.

Comparative analytical data obtained with both plasma and

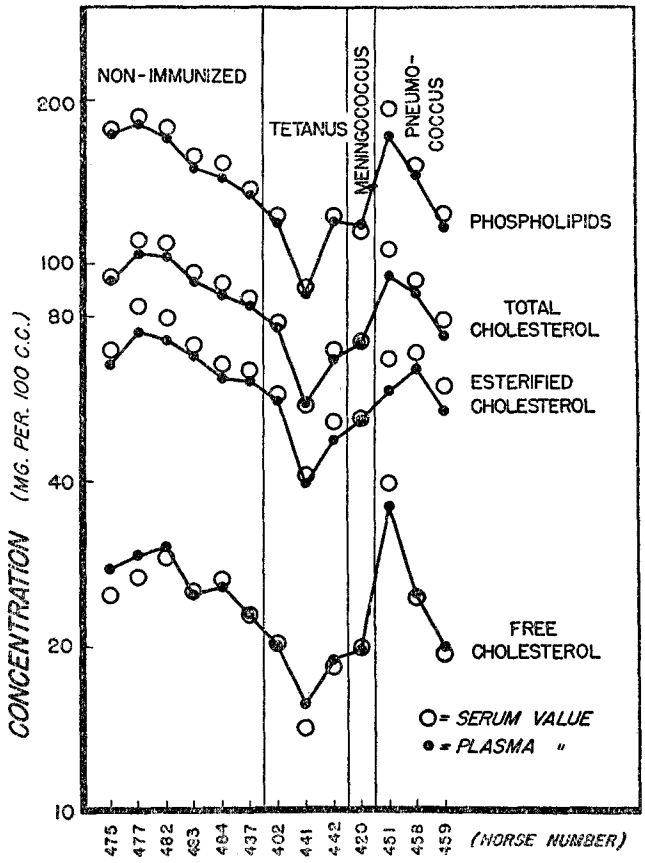

CHART 1. COMPARATIVE ANALYSES OF THE CONSTITUENTS UNDER INVESTIGATION IN SERUM AND PLASMA FROM THE SAME BLEEDING

serum from the same bleeding are given in chart 1. Effects of the use of anticoagulants other than oxalate have not been investigated. Although quantitative variations occur, the ratios between the phospholipids and free and esterified cholesterol are remarkably constant as shown by equal differences between

corresponding points on the logarithmic graph. In the chart, all the black points representing the same constituents are joined to guide the eye.

RESULTS OF ANALYSES

The results of the analyses in the several groups of nonimmunized and immunized horses are recorded on charts 2–6 which have been prepared with the following notes of explanation by Dr. W. R. Thompson.

Estimation of normal ranges: For any given plasma-constituent the central 90-per-cent range for normal horses selected for immunization has been estimated by the rigorous but simple method given in another communication (16). By central is implied equal expectation of values above as below the range in random sampling of the universe (in this case 5 per cent each). The universe consists of observations of the specified type, and, in each case, 62 such observations from different presumably normal horses ready for immunization were available by taking the last observation before antigenic treatment began, or the first of a series of normal bleedings in the case of untreated horses.

In each case the data were considered as a set of $n = 62$ random observations from an infinite universe (the case of a relatively large finite universe gives nearly the same result (16)). If x_k is the k-th of the observed values in order of ascending magnitude, then the expectation that a future random observation (x) be less than x_k is exactly $\frac{k}{n+1}$. The data and values of k for the corresponding arrangements are given in table 1. As 5 per cent $= \frac{3.15}{63}$ and 95 per cent $= \frac{59.85}{63}$, and little difference was found between values for $k = 3$ or 4 in any case, and similarly for $k = 59$ or 60, the required ranges were readily estimated as follows:

	mgm. per 100 cc.
Phospholipids	117.2, 177.3
Free cholesterol	18.8, 30.5
Esterified cholesterol	50.5, 82.5

The data obtained from plasma-analyses during the course of immunization have been represented on semilogarithmic graphs, several of which are given in each chart. Observed values of esterified-cholesterol concentration in the plasma of a given horse are plotted against

TABLE 1

Normal horse plasma phospholipids and cholesterol

(Milligrams per 100 cc.)

HORSE NUMBER	PHOSPHOLIPIDS (P.L.)	FREE CHOLESTEROL (F.C.)	ESTERIFIED CHOLESTEROL (E.C.)	ORDER		
				P.L.	F.C.	E.C.
547	111.9	18.8	51.3	1	3	5
413	116.4	19.1	47.3	2	4	2
472	117.1	23.8	69.9	3	36	48
542	117.8	17.6	53.3	4	2	10
498	118.2	17.2	44.5	5	1	1
421	122.5	20.4	50.5	6	11	3
444	122.6	20.3	51.5	7	10	6
411	124.2	19.9	55.0	8	8	12
155	125.9	22.2	60.5	9	22	21
493	126.3	19.2	50.8	10	5	4
554	129.2	21.4	53.8	11	17	11
546	129.6	22.5	58.9	12	28	17
406	134.1	21.7	52.1	13	19	7
487	134.3	22.9	60.9	14	29	22
380	134.4	21.2	53.3	15	12	9
394	134.9	24.9	61.4	16	43	23
470	134.9	19.4	59.6	17	6	19
409	136.2	23.8	65.3	18	35	36
468	136.6	20.0	66.8	19	9	41
540	137.0	22.3	63.9	20	25	28
429	137.5	21.3	63.9	21	15	27
459	139.9	21.5	59.3	22	18	18
441	142.6	24.3	65.0	23	37	34
484	143.8	25.7	61.6	24	48	24
463	144.3	21.2	68.2	25	13	44
321	144.5	26.4	52.3	26	49	8
500	144.6	21.3	69.1	27	16	46
414	145.0	24.7	67.0	28	41	42
541	145.4	23.4	64.8	29	33	33
548	145.5	25.4	65.1	30	45	35
479	146.2	25.5	62.7	31	47	26
412	146.9	22.1	60.4	32	20	20
469	147.4	22.5	57.8	33	26	14
483	149.9	24.9	67.8	34	44	43
499	150.5	22.1	64.4	35	21	29

TABLE 1—*Concluded*

HORSE NUMBER	PHOSPHO-LIPIDS (P.L.)	FREE CHOLESTEROL (F.C.)	ESTERIFIED CHOLESTEROL (E.C.)	ORDER		
				P.L.	F.C.	E.C.
456	150.7	22.2	64.6	36	23	31
491	150.7	21.2	71.8	37	14	53
437	151.1	23.6	57.1	38	34	13
458	151.1	19.7	58.3	39	7	15
442	151.5	24.3	62.0	40	38	25
460	153.8	24.8	65.6	41	42	39
492	153.8	22.9	64.5	42	30	30
467	153.9	24.3	75.1	43	39	57
407	154.0	23.2	58.4	44	31	16
544	155.2	23.3	71.5	45	32	51
490	157.4	22.5	70.1	46	27	49
447	160.7	25.5	66.7	47	46	40
481	161.8	28.6	69.7	48	53	47
443	162.9	24.4	64.8	49	40	32
501	162.9	22.3	72.4	50	24	55
551	163.2	29.0	70.4	51	56	50
550	164.4	35.6	94.5	52	62	62
555	165.8	28.8	71.5	53	54	52
471	169.2	30.2	81.7	54	58	58
478	169.2	26.8	65.5	55	50	38
482	170.0	30.5	72.4	56	60	54
475	173.6	27.7	65.3	57	52	37
426	176.8	29.0	82.6	58	55	60
392	177.3	31.6	85.0	59	61	61
466	177.3	30.4	82.1	60	59	59
440	180.2	26.9	68.8	61	51	45
477	180.2	29.4	74.9	62	57	56

elapsed time since the initiation to antigenic treatment, and the points are joined to form curve 1. In like manner the corresponding data for free cholesterol and one-tenth the phospholipid concentration form curves 2 and 3, respectively. As these three curves are always distinct and lie in the same relative position in all cases, from top to bottom in the order of enumeration, a single coördinate plane has been used for their representation in the case of each horse, whose number is given below the graph. No further labeling seemed necessary; but, to facili-

tate comparisons,[1] the 90-per-cent central ranges for normal horse plasma have been denoted by pairs of horizontals joined by a bracket at the right of each graph and enumerated (1) esterified cholesterol, (2) free cholesterol, and (3) one-tenth phospholipids. The data for nonimmunized (normal) horses are represented similarly.

The most striking feature of the graphs in general is the slight variation in distance between any two of the three curves for a given

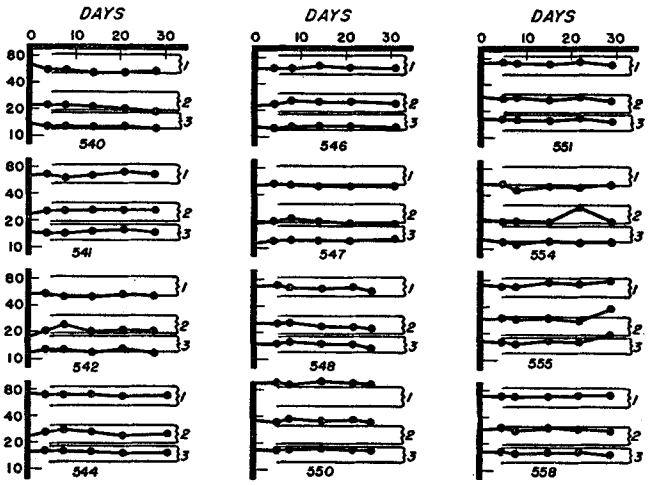

CHART 2. On each graph for a given normal (nonimmunized) horse, the number of which appears below, the curves in descending order respectively represent (*1*) esterified cholesterol, (*2*) free cholesterol, and (*3*) one-tenth phospholipid concentration in plasma (in milligrams per 100 cc.). Corresponding 90-per-cent normal-range estimates are indicated.

animal. This is the principal reason for use of the logarithmic graph. As is well known, the relation, $\log \frac{x}{y} = \log x - \log y$, makes a stable ratio between two variables evident by a correspondingly stable difference between their logarithmic graphs.

Observations on 12 normal (nonimmunized) horses over a period approximating 30 days are presented in chart 2, but the only observa-

[1] Precise statistical tests of the significance of changes in lipid concentrations have been treated separately (17).

tions of this kind protracted over a period corresponding to usual courses of immunization are those for horse 392 given in chart 4. Unfortunately, this horse had next to the highest concentration in the normal experience of 62 values, both for esterified and free cholesterol, and was fourth in phospholipid concentration; but no tendency to decline appeared until after 19 months of observation. However, about two months earlier, thrush was noted and daily treatment instituted for about three and a half months. The association of this with the graph-depressions may be noteworthy.

The difference in time-scales should be borne in mind in comparison of charts 2 and 3 with those following.

It was with the horses injected with diphtheric toxin that the greatest variation in the effect of the toxins was observed under different experimental conditions (chart 3). Acute toxic conditions were observed to be associated with marked changes and irregularities in the lipids, which resulted in changed ratios. This is shown in the case of the three horses that died (nos. 380, 421, and 463), and again with horse 466 which, at the end of 90 days, developed an acute, nearly fatal toxemia following the toxin-injections, which were then abandoned for approximately three months until the animal recovered. This toxemia developed despite the fact that the horse had an antitoxic titer of approximately 1000 units.

The general trends of the more prolonged immunization indicated an early decrease in the lipids followed by a return to the normal range. These trends were apparent also in the other immunized horses. In general, too, the ratios of the phospholipid, free, and esterified cholesterol were practically constant throughout the course of immunization.

In the five horses that responded by producing tetanal antitoxin, the minimal values of phospholipids and cholesterol were reached approximately four months after starting immunization (chart 4). These lipids thereafter increased until approximately the eleventh month and then showed some tendency to decrease. In one horse (442), which responded poorly to antitetanal immunization, the phospholipids and cholesterol decreased only slightly in comparison with the more typically

reactive horses injected with tetanal toxin. The horse was rested for a period of three months and again immunized; four months later the titer was still low—150 units. The phospholipids and the total cholesterol showed relatively little decrease

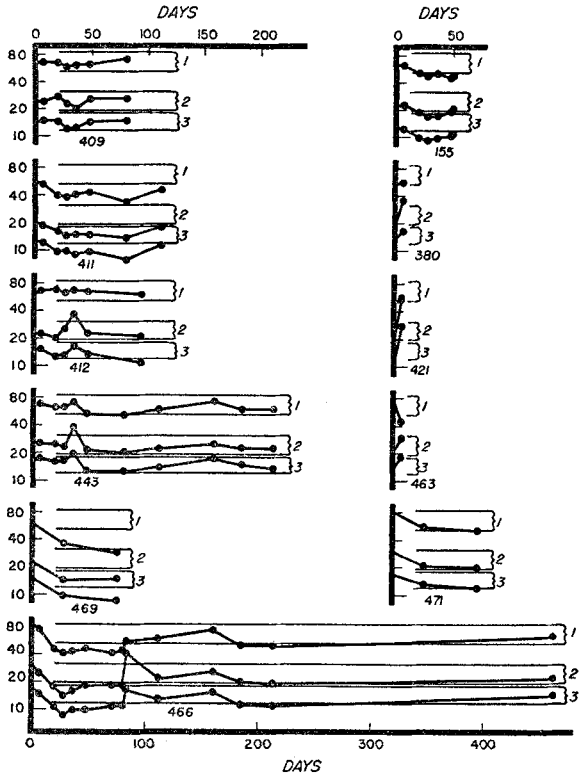

CHART 3. On each graph for a given horse under immunization against diphtheric toxin, the curves in descending order respectively represent (*1*) esterified cholesterol, (*2*) free cholesterol, (*3*) one-tenth phospholipid concentration in plasma (in milligrams per 100 cc.). Corresponding 90-per-cent normal-range estimates are indicated.

when compared with the plasma-analyses previous to immunization. A control on the remote possibility of some influence other than that of the tetanal toxin is furnished by the horses undergoing antipneumococcal immunization, the first five of which

were initiated at about the same date. The initiation-dates are given for convenience in table 2.

The graphs relating to the four horses injected with strepto-

TABLE 2

Dates of initiation of antigenic treatment or serial observation of normal horses

CHART NUMBER	IMMUNIZATION AGENT	INITIATION DATE	TOTAL HORSES	HORSE NUMBER
2	Normal horses	December 9, 1935	6	540, 541, 542, 544, 546, 547
	Normal horses	December 11, 1935	6	548, 550, 551, 554, 555, 558
3	Diphtheric toxin	July 18, 1934	2	469, 471
	Diphtheric toxin	December 14, 1933	9	All others
4	Tetanal toxin	November 17, 1933	3	321, 413, 437
	Tetanal toxin	November 20, 1933	3	441, 442, 444
	Streptococcus	June 4, 1934	2	414, 429
	Streptococcus	June 25, 1934	2	460, 467
	Normal horse	October 20, 1933	1	392
5	Meningococcus	March 13, 1935	2	442, 443
	Meningococcus	July 5, 1934	3	468, 470, 472
	Meningococcus	February 5, 1935	2	475, 477
	Meningococcus	March 4, 1935	1	482
	Meningococcus	February 8, 1935	3	483, 484, 487
	Meningococcus	March 13, 1935	2	490, 493
6	Pneumococcus I	November 24, 1933	2	406, 407
	Pneumococcus I	October 31, 1933	3	456, 458, 459
	Pneumococcus VIII	February 2, 1934	1	440
	Pneumococcus II	March 14, 1935	1	469
	Pneumococcus II	January 7, 1935	1	478
	Pneumococcus II	December 11, 1934	1	481
	Pneumococcus II	March 13, 1935	2	491, 492
	Pneumococcus I	March 28, 1935	4	498, 499, 500, 501

cocci show a definite lowering of the phospholipids and cholesterol in the plasma during immunization (chart 4).

Thirteen horses receiving meningococci were studied and the lipids of the plasma showed an early tendency to decrease (chart 5).

With the plasma samples from the horses injected with pneumococci, the irregular increases and decreases in the phospholipids were not sufficient to indicate a general trend (chart 6). In fact, they approximated the normal range, although some effects

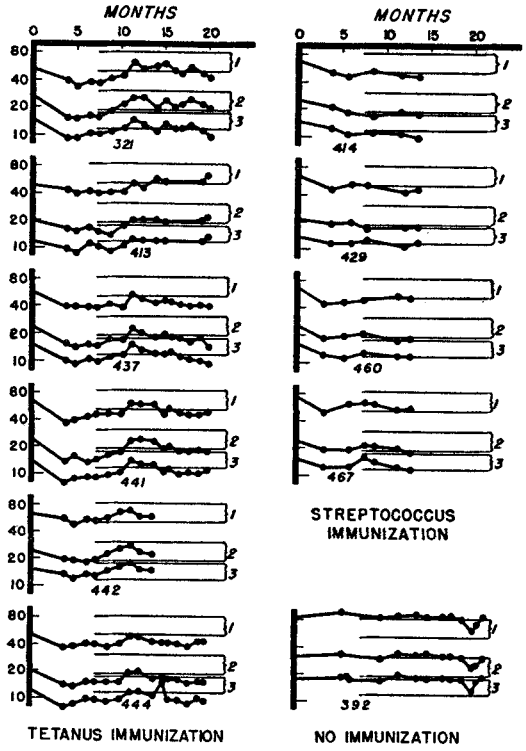

CHART 4. On each graph for horses under immunization against the indicated antigen and one nonimmunized horse, the curves in descending order respectively represent (*1*) esterified cholesterol, (*2*) free cholesterol, and (*3*) one-tenth phospholipid concentration in plasma (in milligrams per 100 cc.). Corresponding 90-per-cent normal-range estimates are indicated.

of the immunization might be found if further study were made at short intervals under more acutely toxic conditions.

DISCUSSION

It is difficult to study the results from the standpoint of immunization by intravenous as contrasted with subcutaneous in-

jection because the effects of the powerful diphtheric and tetanal exotoxins given subcutaneously overshadow the effects of intravenous injection of the bacterial cultures either living or dead. The fluctuations of phospholipids and esterified and free cholesterol, and their ratios, in the plasmas of the twelve uninoculated horses correspond approximately to the normal ranges deter-

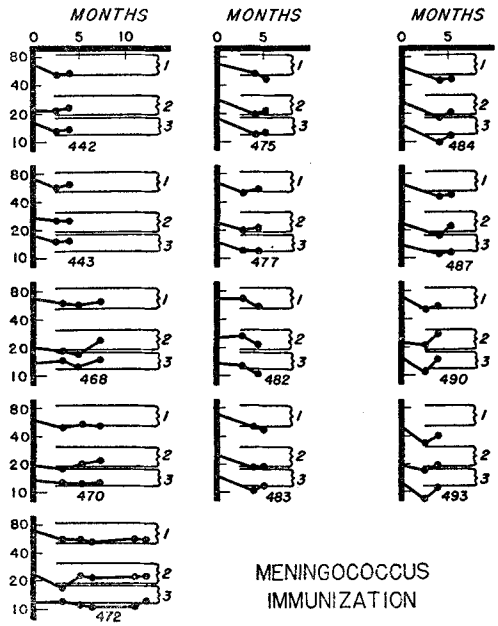

CHART 5. On each graph for a given horse under immunization against the meningococcus, the curves in descending order respectively represent (*1*) esterified cholesterol, (*2*) free cholesterol, and (*3*) one-tenth phospholipid concentration in plasma (in milligrams per 100 cc.). Corresponding 90-per-cent normal-range estimates are indicated.

mined from analyses of the 62 independent plasmas from untreated horses (table 1). An individual horse at the time of any particular bleeding, however, may or may not have been in a perfectly healthy or normal condition. Changes in the different lipids during immunization are approximately proportional and their ratios fairly constant, with few exceptions such as those that occurred in the case of the horses with acute diphtheric

toxemia. Although the main trends—decrease during the early period followed by return to normal range—were apparent in most of the processes of immunization, the depressions varied in degree: they were most marked with exotoxins, practically absent in the horses receiving pneumococci.

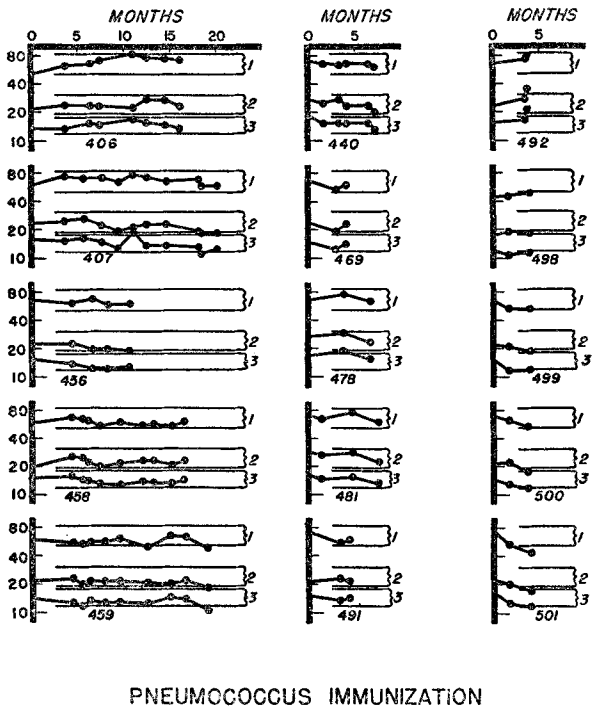

CHART 6. On each graph for a given horse under immunization against the pneumococcus, the curves in descending order respectively represent (*1*) esterified cholesterol, (*2*) free cholesterol, and (*3*) one-tenth phospholipid concentration in plasma (in milligrams per 100 cc.). Corresponding 90-per-cent normal-range estimates are indicated.

The circulating blood appears to maintain an equilibrium in its phospholipid content under normal conditions as well as during the processes of immunization used in these studies. Even when there is a marked decrease or increase in the phospholipid content, the adaptive capacity of the animal tissues tends to and ultimately does maintain a stable balance in the

relationships of the phospholipids and cholesterol in the plasma. This is particularly manifested by results obtained in the analyses of horse 466 (diphtheria) during and following the acute toxemia that occurred toward the end of the third month.

The changes in the lipids of the liver recorded in our previous report corresponded with the injurious effects of the various processes of immunization. In general, within certain limitations, the reactions of immunization that follow injury vary directly with the extent of that injury, or indirectly if these limits are exceeded. It has long been recognized that pneumococcal lesions are characterized by an exudative process in which little or no necrosis of the tissues occurs; the resolution of lobar pneumonia, for example, is practically complete without scar tissue or evidence of damage. Although the character of streptococcal infection may vary widely, the tissues are usually more severely injured than in pneumococcal infection. With the meningococcus, the injection of culture-material is often followed by markedly toxic manifestations. Diphtheric toxin, when injected into the tissues, is invariably associated with necrosis. The severity of injury induced by the different antigens injected in these animals doubtless exerted a powerful influence in the production of observed results. Moreover, the effect of the localized process obtained with subcutaneous injection in contrast with the more general action that follows intravenous injection, may account for some of the differences observed in the effects on lipid concentrations.

SUMMARY

Variations in the concentrations of phospholipids and of free and esterified cholesterol in the plasma and serum of horses previous to and during immunization with diphtheric and tetanal toxins, and with meningococci, streptococci, and pneumococci are recorded.

The fluctuations in the different lipids during immunization were approximately proportional and their ratios fairly constant, except in certain horses with acute diphtheric toxemia.

The main trends in the process of immunization were a de-

crease in the lipid content during the early period followed by a return to the normal range. The depressions, however, varied in degree and were most marked with the exotoxins, practically absent in the horses injected with pneumococci.

REFERENCES

(1) WADSWORTH, A. B., HYMAN, L. W., AND NICHOLS, R. R.: Amer. Jour. Path., 1935, **11**, 419.
(2) MARIE, A.: C. r. Soc. de biol., 1923, **88**, 76.
(3) MARIE, A. C.: Ann. de l'Inst. Pasteur, 1923, **37**, 921.
(4) MARIE, A. C.: Ann. de l'Inst. Pasteur, 1924, **38**, 945.
(5) DANYSZ-MICHEL, AND LASKOWNICKI, ST.: C. r. Soc. de biol., 1924, **91**, 632.
(6) DAMBOVICEANU, A.: C. r. Soc. de biol., 1929, **101**, 325.
(7) IONESCO-MIHAIESTI, C., AND DAMBOVICEANU, A.: C. r. Soc. de biol., 1930, **104**, 1357.
(8) FELTON, L. D., AND KAUFFMANN, G.: Jour. Immunol., 1933, **24**, 543.
(9) HYMAN, L. W., AND NICHOLS, R. R.: Jour. Bact., 1937, **33**, 110.
(10) WHEELER, M. W.: In: Ann. Rep. Div. Labs. and Research, N. Y. State Dept. Health, 1934, 89 pp.
(11) KIRKBRIDE, M. B., BERTHELSEN, K. C., AND CLARK, R. F.: Jour. Immunol., 1931, **21**, 1.
(12) WADSWORTH, A. B.: Standard Methods of the Division of Laboratories and Research of the New York State Department of Health. Baltimore, The Williams & Wilkins Company, 1927.
(13) BLOOR W. R.: Jour. Biol. Chem., 1928, **77**, 53.
(14) PREGL, F.: Quantitative Organic Microanalysis. 3d English edition. Philadelphia, P. Blakiston's Son & Company, Inc., 1937.
(15) OSATO, S., AND HEKI, M.: Jour. Biol. Chem., 1930, **87**, 541.
(16) THOMPSON, W. R.: Ann. Math. Statistics, 1936, **7**, 122.
(17) THOMPSON, W. R.: Biological applications of normal range and significance estimates in ignorance of original distribution forms. Presented before Annual Meeting of Amer. Statistical Assoc., Dec. 28, 1937, Atlantic City, N. J.