SOME VIRAL, RICKETTSIAL AND LEPTOSPIRAL INFECTIONS DIAGNOSED IN SERBIA. A SEROLOGICAL STUDY

By A. L. TERZIN,* M. N. BORDJOŠKI, M. V. MILOVANOVIC,
LJ. V. STOJKOVIĆ AND M. M. DIMIĆ

*The Department of Virology, Institute of Hygiene, Belgrade,
and the Yugoslav Regional Centre for Influenza, Belgrade*

(With 1 Figure in the Text)

The newly founded Virology Department of the Institute of Hygiene in Belgrade started to develop regular routine diagnostic work on 1 January 1952. By 31 March 1953, 9370 serological tests had been performed for various viral and rickettsial infections. The results of these tests are presented in this paper.

The following serological tests have been used for examining specimens: the complement-fixation reaction with various viral and rickettsial antigens, the cold-agglutination reaction, the agglutination of streptococcus MG, the Paul-Bunnell reaction and the agglutination-lysis test for leptospirosis.

On some specimens from patients with influenza and mumps infections, inhibition of haemagglutination reactions have been performed in addition to the more usual serological procedures.

The well-known similarity of the clinical conditions caused by some viral infections with those caused by some leptospiral infections necessitated simultaneous and joint work with both viral and leptospiral antigens.

SOURCES, QUALITY AND NUMBER OF THE SERA SENT FOR TESTING

We have undertaken neither field work nor epidemiological investigations, and no analysis of the territorial distribution of the positive cases has been attempted. The specimens sent to us consisted of the following two groups of sera:

(1) Specimens sent from different clinics and hospitals in Belgrade and rarely also from institutions in the countryside of Serbia.

Some of the physicians have repeatedly sent us specimens to be investigated 'for virus infection', although many of these specimens originated from afebrile patients who should never have been suspected of virus infections. The Ophthalmology Department has regularly sent sera to us to be investigated systematically for leptospirosis and more recently for toxoplasmosis as well.

Only a small proportion of the specimens submitted for examination originated from patients suffering from acute febrile disease whose nature could not be determined clinically and thus were sent to us in order that we might confirm or exclude a justifiable suspicion of certain viral or rickettsial infections.

(2) Specimens collected by epidemiologists in the field and submitted for systematic examination. Most of these sera had been collected from normal persons

* Present address: The Department of Virology, Medical Faculty, Sarajevo.

dwelling in epidemic areas, from convalescents or from animals in Serbia and Bosnia.

It is evident that only a small minority of the specimens submitted to our laboratory represented material on which the search for viral infection had a fair chance of success.

Altogether 3500 specimens of human serum originating from 2430 patients have been tested in the period of this investigation which covered 15 months.

About three-quarters of all the sera tested were collected from patients in the course of the first month or their illness. Table 1 records the percentage of serum samples collected at different times from the onset of disease.

Table 1. *The percentage of serum samples collected at different intervals from the date of onset of disease*

Time from onset of disease to collection of serum ...	1-5 days	6-14 days	15-30 days	31-60 days	61-90 days	4-6 months	7-12 months	More than 1 year and unknown
Distribution percentage of sera collected on different days	13	32	32	9	5	4	2	3

Table 2. *An analysis of the number of blood samples taken from individual patients*

	No. of blood samples taken from single patients								Totals
	1	2	3	4	5	6	7	More than 7	
No. of patients	1757	444	141	48	20	9	6	5	2430
No. of blood samples	1757	888	423	192	100	54	42	44	3500

Table 2 presents some information on the average number of blood samples drawn from single patients, it shows that about 50 % of the sera tested were single specimens. As many as ten samples of blood were taken from some of the patients while they were under observation.

Syndromes

Each of the 3500 samples of human sera submitted for laboratory examination had been provisionally diagnosed for clinical reasons as from one of the following seven syndromes:

(1) *Pyrexia of unknown origin*. This formed the largest group of specimens for we have included in it all the sera originating from acute febrile patients which did not fit into any of the other six syndromes listed in Table 3. This group contained specimens from patients who had been diagnosed as suffering from status febrilis, parotitis, angina, encephalitis, and other syndromes.

(2) *Hepatitis*. This group of sera was made up of specimens taken from patients diagnosed by the clinicians as infective hepatitis, serum jaundice, icterus hepatocellularis, hepatitis parenchymatosa, etc. We have not refused to perform a cold-

agglutination test on these sera and often also a streptococcus MG agglutination, because raised titres in these two tests have been observed several times in cases of liver damage. This will be mentioned again later. However, we have always emphasized to the physicians the fact that cold agglutination can only be used for the diagnosis of a certain atypical virus pneumonia and that in cases of liver disease it has no diagnostic value whatsoever.

During the last 2-3 years, a very high incidence of both infective hepatitis and serum jaundice has appeared in Belgrade. After the first positive diagnosis of leptospirosis many physicians sent us sera from all their jaundice cases to be tested for leptospiral infection, a fact that may be responsible for the large number of sera submitted with the diagnosis 'hepatitis'.

Table 3. *An analysis of 3500 samples of human serum sent for investigation under seven different clinical diagnoses*

Clinical diagnosis	No. of sera tested	No. of sera found positive								Total
		Lepto-spirosis	Q-fever	Mumps	Infectious mononu-cleosis	Influenza A and B	Typhus	Atypical virus pneu-monia	Lympho-cytic chorio-meningitis	
Pyrexia of un-known origin	869	14	6	12	26	—	—	—	—	58
Hepatitis	660	—	—	—	—	—	—	—	—	0
Miscellaneous	650	—	—	—	—	—	—	—	—	0
Pneumonia	409	—	16	—	—	14	—	50	—	80
Meningitis	393	27	—	16	—	—	—	—	8	51
Grippe	329	—	—	—	—	92	—	—	—	92
Status typhosus	190	—	5	—	—	—	46	—	2	53
Totals	3500	41	27	28	26	106	46	50	10	334

(3) *Miscellaneous*. This group included all the sera from afebrile cases which were sent to us to be tested for 'viruses', for cold agglutinations, etc. This group included also a large number of sera, to be mentioned later, which were sent to us from patients in the ophthalmological departments.

(4) *Pneumonia*. The syndrome pneumonia included cases of bacterial pneumonia as well as those of viral and rickettsial origin which were diagnosed clinically as atypical virus pneumonias. As stated by Terzin (1952) we find it very important to distinguish between the syndrome of atypical virus pneumonia which may be caused by any one of a number of filtrable agents such as Q-fever, psittacosis, influenza, Eaton's SF virus, etc., and the disease of atypical virus pneumonia caused by a certain agent or agents related to Eaton's SF virus and usually giving rise to the formation of cold agglutinins.

(5) *Meningitis*. All the sera taken from patients clinically diagnosed as suffering from meningitis are grouped under this syndrome. This group contained sera from patients with meningitis caused by *Neisseria meningitidis* and other micro-organisms as well as from cases of lymphocytic meningitis caused by *Mycobacterium tuberculosis*, leptospirae and various viruses.

(6) '*Grippe*' syndrome. All sera from patients diagnosed clinically as 'grippe' have been grouped under this heading. Patients who developed acute infection

of the upper respiratory tract, cases of common cold, febrile catarrh, cases of acute infection with some encephalitis virus, etc., were probably responsible for those cases clinically diagnosed as 'grippe' which proved not to be influenza infections.

(7) *Status typhosus*. Many of the symptoms which typhus, typhoid and relapsing fevers have in common, may also be pronounced in some cases of Q-fever, lymphocytic choriomeningitis, psittacosis, dengue, virus encephalitis or leptospirosis.

Table 3 shows the distribution of the 3500 human sera from 2430 patients, according to the syndromes just described, with a brief review of the positive findings. The highest incidence of positive results has been obtained in the syndromes of 'grippe' and status typhosus, next follow pneumonia and meningitis and the lowest incidence of positive results has occurred in the syndrome pyrexia of unknown origin. Of the 1310 sera grouped under the syndromes hepatitis and miscellaneous not a single one has given a positive result.

Usually the material to be tested was submitted to the laboratory, with a tentative diagnosis of the syndrome. Sometimes, however, it happened that the laboratory had to correct a definite diagnosis given by the physician. For example, we have seen cases clinically diagnosed as 'typhus fever' which after laboratory tests turned out to be Q-fever or lymphocytic choriomeningitis infections, while some cases diagnosed clinically as 'LCM-meningitis' were shown to be infections with leptospirae or mumps virus and some of the sera drawn from patients diagnosed clinically as 'leptospirosis' have been diagnosed by laboratory tests as Q-fever or LCM-virus infections.

Scheme for screen testing the material

Table 4 presents the scheme we have used for the initial screen testing of the sera.

In the initial screen tests for influenza A and B, mumps, typhus and Q-fever, the sera have been tested in dilutions of 1/8 and 1/32 using complement-fixation reactions both with and without the specific antigens. We have tested these sera in dilutions of 1/8 and 1/32 instead of the more usual dilutions of 1/8 and 1/16, because of the occurrence of prozones which have sometimes been observed to reach values as high as 1/16.

Sera to be tested for lymphocytic choriomeningitis have been tested in dilutions from 1/2 to 1/32 for complement fixation both in the presence of specific antigen and without it, as well as in the presence of normal spleen extract.

For the Paul-Bunnell reaction, the sera have been tested first unabsorbed in dilutions of 1/10 to 1/640 and only those agglutinating the sheep-cell suspension to titres of 1/40 or higher were absorbed with the guinea-pig spleen suspension and retested with sheep cell suspension.

The streptococcus MG agglutination reaction has been performed with serum dilutions of 1/10 to 1/160 without a preliminary rough screen test.

No screen test has been used when testing for cold agglutinins. A full series of dilutions from 1/16 to 1/2048 has been made for the initial testing of the serum.

Screen testing of the sera for antibodies to leptospirae was performed at a dilution of 1/30, except when testing for antibodies to *Leptospira sejroe* when an additional serum dilution of 1/100 was used as this strain, in our hands, tended to show a prozone at the 1/30 serum dilution.

Table 4. *The scheme used for the initial 'screen-testing' of the sera based on the clinical diagnosis*

Clinical diagnosis	Sera screen-tested for
Aseptic meningitis	Lymphocytic choriomeningitis, mumps, infective mononucleosis, leptospirosis, toxoplasmosis
Encephalitis-myelitis	Mumps, influenza, leptospirosis, toxoplasmosis
Grippe	Influenza, atypical virus pneumonia, lymphocytic choriomeningitis, Q-fever, leptospirosis
Atypical virus pneumonia	Atypical virus pneumonia, Q-fever, leptospirosis, lymphocytic choriomeningitis, toxoplasmosis
Eruptions on skin and mucous membranes	Typhus fever, leptospirosis, toxoplasmosis and rickettsial pox
Lymphadenopathia	Infective mononucleosis, toxoplasmosis
Status typhosus	Lymphocytic choriomeningitis, Q-fever, influenza, leptospirosis, toxoplasmosis and rickettsial pox

Testing for toxoplasmosis was only introduced towards the end of the survey.

When testing for atypical virus pneumonia both cold agglutination and streptococcus MG agglutination tests were made.

Serological tests

(1) Complement-fixation reaction

Antigens. The following antigens have been used: the soluble antigens of influenza A and B made from the infected chorioallantoic membranes of fertile eggs as recommended in the Report (1953); mumps antigen made from the allantoic fluid of mumps-infected fertile eggs; LCM antigen (soluble type), made from infected guinea-pig spleens according to the method of Smadel, Baird & Wall (1939), but treated with chloroform as recommended by Dr F. O. MacCallum (personal communication); normal guinea-pig spleen extracts; toxoplasma antigen made from the thrice-frozen and thawed sediment of the peritoneal exudate of mice infected with strain RG-Sabin, diluted to 100 mg/1 ml. saline. All six antigens were prepared in our laboratory. The following four commercial antigens made by Lederle Labs. U.S.A., have also been used: Q-fever (Henzerling), epidemic and murine typhus soluble antigens and mumps viral antigen. All antigens have been checkerboard-titrated with known positive sera and checked for anticomplementary as well as for haemolytic activity. The influenza, mumps, LCM and toxoplasma antigens have been used for the diagnostic test in the dilution which showed the optimal peak in the checkerboard titration. The unit dilution of other antigens has been determined by titrating the antigen in the presence of eight antibody units of its specific anti-serum. Two units of the respective antigen were

used in the diagnostic test. The sera to be tested were first inactivated in a water-bath at 57° C. for 30 min. at the lowest dilution which was to be used in the test.

Other reagents. Guinea-pig serum preserved by the method of Richardson (1941) has been used as complement. The haemolysin has been prepared and titrated in this laboratory. The diluent used for all the tests was a 0.85 % solution of sodium chloride.

Method of titration. Sera to be tested with LCM and toxoplasma antigens were serially diluted from 1/2. Sera against all other antigens were tested in twofold serial dilutions starting from 1/8. The complement-fixation technique used was Hoyle's 'small volume test' (Hoyle, 1948) as modified by the Virus Reference Laboratory, Colindale (B. E. Andrews; in a personal communication). The unit volume used was 0.1 ml. The complement was titrated against each batch of sensitized cells (2 % cells with 2 units of haemolysin) and the two units of complement used in the test were strictly controlled by control tubes set up to contain two, one, half and a quarter of a unit of complement.

With all antigens, except LCM, we have used a 90 min. period of fixation at 37° C. and, after the addition of the sensitized red cells, an incubation period of 30 min. With LCM antigen an overnight fixation at +4° C. was used.

Controls of known positive and negative sera with the antigen, controls of the antigen and of the sera to be tested without antigen and in the three lowest dilutions used, and controls of the exact amount of complement used were included in each set of tests. The results were disregarded and the test repeated the next day, if any of the controls proved unsatisfactory.

(2) *Paul Bunnell reactions*

The technique used was that described by Barrett (1941).

(3) *The cold agglutination reaction*

The technique used was that described by Terzin (1950). Sera to be tested for cold agglutinins have been separated from their blood clots after subjecting them for about 20 min. to a thermostatically controlled temperature of 37° C. The separated sera have been tested in twofold serial dilutions, the dilutions were made with saline in 0.5 ml. volumes and made up to a volume of 1 ml. by adding to each dilution 0.5 ml. of a 0.5 % suspension of human group O red blood cells. The final concentration of the red blood cells in each tube was thus about 20,000 per cu.mm. After incubation for 3 hr. at +4° C. the agglutination was read from the bottom pattern by viewing, without previous shaking.

(4) *The streptococcus MG agglutination reaction*

The technique used was that described by Thomas, Mirick, Curnen, Ziegler & Horsfall (1945).

(5) *The agglutination-lysis test for leptospirae*

All sera have been tested in serial dilutions of about 3.3-fold by means of a modification of the technique described by Schüffner & Mochtar (1927) as performed in the Staatens Serum Institute, Copenhagen (Ch. Borg Petersen; in

a personal communication). As a rule each serum has been tested against the following eight strains: *Leptospira icterohaemorrhagiae* AB (complete); *L. canicola* UIV; *L. sejroe* M₈₄; *L. grippotyphosa* Schlesien; *L. pomona* Pomona; *L. mitis* Johnson; *L. batavia* van Thienen and *L. australis* A. Ballico. Exceptionally sera have been tested also with the following two strains: *L. sachskoebing* and *L. icterohaemorrhagiae* Kantorovitz (incomplete).

RESULTS AND CRITERIA ON WHICH THE RESULTS WERE BASED

Table 5 shows the distribution of our results over the five 3-month periods of this investigation.

Positive results were diagnosed only if there was at least a fourfold or more (tenfold with leptospira) rise of titre.

Occasionally a positive diagnosis has been based on a single test provided that this gave a high titre and that the serum had been obtained from a case which clinically and epidemiologically was very suggestive of a particular disease.

The reactions used in making the diagnoses and the critical titres beyond which it was considered necessary that the test should go before a positive diagnosis was made were as follows. Influenza was diagnosed by a complement-fixation reaction positive to a titre of at least 1/64; atypical virus pneumonia by titres of at least 1/128 in the cold agglutination reaction and 1/40 in the strep. MG agglutination reaction provided these results agreed with the clinical findings; Q-fever was diagnosed by a complement-fixation reaction positive to a titre of at least 1/64. Complement-fixation reactions were also used for the diagnosis of typhus fever when the titre was greater than 1/64; mumps when the titre was greater than 1/64; lymphocytic choriomeningitis when the titre was greater than 1/8. Infectious mononucleosis was diagnosed by a Paul-Bunnell reaction with a titre of 1/40 or more and leptospirosis by an agglutination-lysis reaction to a titre of 1/1000 or more.

ANALYSIS OF THE RESULTS

Table 6 shows that a total of 8741 tests have been performed on human sera in the period of 15 months. The agglutination-lysis reaction performed with eight to ten strains of leptospirae has been counted as a single test and the complement-fixation reaction with influenza A and B antigens as two separate tests.

Analysis of the negative results gives some information about the titres to be expected in the 'normal' population. Without a knowledge of these values it would be difficult to develop a successful and reliable serological diagnosis of viral and rickettsial infections.

Besides the 3500 samples of human sera, we have also investigated 536 animal sera, usually as single specimens tested for leptospirosis or Q-fever or both. Tables 17 and 27 summarize the results of the tests performed with these animal sera. Table 7 records the number of tests performed, the number of sera tested, as well as the number of positives found both in the human and animal material tested by us.

Table 5. *An analysis of results based on the five 3-month periods of this investigation*

Laboratory diagnosis	Clinical diagnosis	No. of patients						Sub-total	Total	No. of sera
		3-month periods								
		I	II	III	IV	V				
Influenza A	Grippe	—	1	—	—	87	88	106	173	
Influenza B	Pneumonia	—	—	—	—	12	12			
	Grippe	3	—	—	1	—	4			
	Pneumonia	2	—	—	—	—	2			
Atypical virus pneumonia	+ cold agglutination + strep. MG	Pneumonia	2	1	1	—	3	7	50	105
	— cold agglutination + strep. MG	Pneumonia	1	—	—	1	2	4		
	+ cold agglutination — strep. MG	Pneumonia	5	4	2	9	6	26		
+ cold agglutination strep. MG not tested	Pneumonia	2	1	1	2	7	13			
Q-fever	Pneumonia	2	9	3	—	2	16	27	74	
	Status typhosus	1	3	—	—	1	5			
	Pyrexia of unknown origin	—	3	2	—	1	6			
Typhus	Status typhosus	4	13	7	11	11	46	46	64	
Leptospirosis	Pyrexia of unknown origin	2	3	7	2	—	14	41	103	
	Meningitis	1	4	11	11	—	27			
Lymphocytic choriomeningitis	Meningitis	2	1	—	4	1	8	10	17	
	Status typhosus	—	1	—	1	—	2			
Mumps	Parotitis	—	8	—	2	1	11	28	52	
	Orchitis	—	—	—	—	1	1			
	Meningitis	2	2	2	1	6	13			
	Parotitis and meningitis	—	1	—	—	2	3			
Infective mononucleosis	Monocytic angina	—	5	6	10	5	26	26	50	
Totals								334	638	

Examples of possible double infections

Out of the five cases diagnosed to be influenza B infections which occurred sporadically between January and March 1952, two appeared clinically to be atypical virus pneumonias. The peak titres of cold agglutinins, MG agglutinins and of influenza B complement-fixing titres in these cases were as follows:

Case no. 1. Cold-agglutinin titre 1/1024, MG-agglutinin titre 1/20, influenza B complement-fixing titre 1/128.

Case no 2. Cold-agglutinin titre 1/128, MG-agglutinin titre 1/40; influenza B complement-fixing titre 1/128. All these reactions showed a fourfold or greater rise of titre during the course of the disease.

Table 6. *An analysis of the tests done on all the sera which were of human origin*

Serological tests	Human sera originating from 2430 patients									Totals
	Lept.	Infl.	Q-fv.	Cold	MG	Mump.	Typh.	P.B.	LCM	
No. of tests performed	3080	1723	1123	997	482	417	381	316	222	8741
No. of sera tested	3080	909	1123	997		417	381	316	222	3500
No of patients found positive	41	106	27	50		28	46	26	10	334
No. of sera taken from positive patients	103	173	74	105		52	64	50	17	638
Lept.	Agglutination-lysis test with eight strains of leptospirae.									
Infl.	Complement-fixation test with influenza A and B antigens.									
Q-fv.	Complement-fixation test with Q-fever antigen.									
Cold	Cold agglutination reaction.									
MG	Strep. MG agglutination reaction.									
Mump.	Complement-fixation test with mumps antigen.									
Typh.	Complement-fixation test with typhus antigen.									
P.B.	Paul-Bunnell reaction.									
LCM	Complement-fixation test with LCM virus antigen.									

Table 7. *An analysis of the tests done on all sera, irrespective of whether they were of human or animal origin.*

	Human	Animal	Totals
No. of tests performed	8741	629	9370
No. of sera tested	3500	536	4036
No. of individuals found positive	334	153	487
No. of sera taken from positive individuals	638	153	791

Of the twelve pneumonias occurring during the influenza A' outbreak between January and March, 1953, at least three were diagnosed by the clinicians as atypical virus pneumonia, on the basis of the white blood cell picture, drug resistance and characteristic pulmonary findings, but they also showed high titres of cold agglutinins, accompanied by rising titres of MG agglutinins and high titres ranging from 1/128 to 1/1024 for influenza A' antigen.

All these patients had a protracted and severe attack of the clinical disease. In all these cases Q-fever was excluded by negative serological findings.

During the influenza outbreak in Belgrade of January to March 1953 we had two specimens of sera from a patient whom we arbitrarily put in the group of influenza A' infections, although developing a significant rise of antibodies both for influenza A' and influenza B, suggesting a double infection with both viruses. The influenza titres of the two specimens of sera taken 7 days apart showed the following increase: with antigen A from 1/16 to 1/128, and with antigen B from negative to 1/16.

Influenza infections

Table 8 shows the results of testing 909 samples of sera with influenza antigens. Of these 909 samples of sera, 802 were tested with A' antigen and 748 with B antigen. Of the same 909 sera, 173 were from cases diagnosed as influenza. All these 173 samples of sera were tested both with A and B antigens, but in Table 8 only the homologous titres of the sera taken from influenza-positive patients are shown. The total number of tests performed with both A and B antigens actually amounted to 1723, but only 1550 are recorded in Table 8.

Table 8. *The homologous antibody content of all sera tested for influenza*

	Anti-gens	Reciprocals of the titres					Total no. of	
		< 8	8-16	32	64	> 64	Tests	Sera
Sera of influenza A patients	A	34	10	16	35	66	161	161
Sera of influenza B patients	B	1	2	2	3	4	12	12
Sera of patients with infections other than influenza	{ A	498	101	42		0	641	736
	{ B	627	105	4		0	736	
Total		—	—	—	—	—	1550	909

Table 9. *Variations in the antibody titres against influenza antigens with the number of days from the onset of disease*

	Days of the disease				Total
	1-7	8-28	29-60	> 60	
No. of the sera tested	49	116	6	2	173
Geometrical means of the homologous titres in influenza infections	1/2.5	1/119	1/79	1/64	1/38

Table 10. *Sera from influenza patients grouped according to their homologous titres from 1/128 upwards*

	Reciprocals of the titres found						Total
	< 128	128	256	512	1024	2048	
No. of sera from influenza patients tested with homologous antigens	103	39	17	5	6	3	173

The distribution of the homologous titres in influenza infected patients showed no significant differences depending on the type of the virus causing the infection. The residual titres found in the blood samples of patients suffering from infections other than influenza, showed much lower levels for influenza B than for influenza A, probably due to the fact that many of these sera were tested in the period when a large influenza A' outbreak swept through the country.

Table 9 shows how the titre of homologous antibodies to influenza virus varied with the period of duration of the disease.

The distribution of the homologous titres higher than 1/64 in influenza infections is presented in Table 10.

Atypical virus pneumonia. On the basis of 105 serum tests fifty patients have been diagnosed as suffering from atypical virus pneumonia. Four of the fifty patients showed a negative cold-agglutination reaction. These sera which gave

negative cold-agglutination reactions as well as the serum samples obtained from those patients before or after the phase of their illness when the cold-agglutination reaction was positive are all included in the 105 sera recorded in Table 11.

Reference to Table 5 shows that forty-six out of fifty of the atypical virus pneumonia patients have been found cold-agglutinin positive according to our criteria. Table 12 shows that the distribution of the titres in the 105 serum specimens originating from the fifty positive patients was such that 58 % of these serum samples showed a cold-agglutination reaction to titres of 1/128 or higher.

Table 11. *Sera from patients with atypical virus pneumonia grouped according to their content of cold agglutinins*

Reciprocals of the cold-agglutinin titres	<8	8	16	32	64	128	256	512	1024	Over 1024
No. of specimens (total of 105 specimens)	3	1	6	13	21	30	14	7	8	2

Table 12. *The correlation of the clinical diagnosis and the cold-agglutinin titres which were found in the patients' sera*

Diagnosis	Sera showing cold agglutinin titres		No. of sera tested
	1/64 or less (%)	1/128 or more (%)	
Atypical virus pneumonia	42	58	105
Mumps	81	19	31
Q-fever	82	18	39
Miscellaneous	84	16	201
Hepatitis	85	15	551
Influenza, typhus, lymphocytic choriomeningitis and leptospirosis	94	5.4	70

The peak titres have been reached mostly in the course of the 3rd week of the illness. Exceptionally we have seen titres of 1/512 as early as the first week of the illness and titres of 1/128 as late as the third month of the illness.

The cold agglutination reaction gave us positive results also with certain sera of patients developing other diseases or diagnosed as diseases other than atypical virus pneumonia.

Table 12 shows the data pertaining to about 1000 serum specimens tested for cold agglutinins. The data are arranged to correlate the clinical diagnosis with the titres of cold agglutinins found, taking the titre 1/128 as critical. The eventual rise or fall of the titres of the sera from those patients suffering from diseases other than atypical virus pneumonia has not as a rule been followed.

Of thirty-one sera tested for cold agglutinins and originating from cases diagnosed as mumps, we have found six with titres of 1/128 or higher.

A surprisingly high incidence of positive cold-agglutination reactions has been found in sera from cases of different varieties of hepatitis such as infectious hepatitis, serum jaundice, etc., as well as in about 15 % of the sera grouped under the heading of 'Miscellaneous'.

The seventy specimens of sera originating from cases diagnosed as influenza, leptospirosis, lymphocytic choriomeningitis or typhus fever, revealed when tested for cold agglutinins a relatively very low incidence of positive reactions of about 6 % of the sera showing titres of 1/128 or higher, and this percentage incidence was about equal for each disease.

The thirty-nine serum samples obtained from Q-fever patients showed an unexpectedly high incidence of about 18 % of cold-agglutinin titres in the range of 1/128 or higher. This observation, although based on a small number of cases, may well indicate the necessity of performing the complement-fixation reaction with Q-antigen in all sporadic cases developing the atypical virus pneumonia syndrome, instead of relying on a positive cold-agglutination test when diagnosing atypical virus pneumonia. We consider that this should be stressed when smaller laboratories which are performing cold-agglutination reactions but are not doing Q-fever tests, intend to call on the services of the central virus laboratory.

The second test used in diagnosing atypical virus pneumonia was the agglutination reaction using streptococcus MG. The results of this test, performed on about 500 serum samples, are presented in Table 13, put in order either according to the titres and syndromes or according to the diagnoses confirmed by the laboratory.

Of the fifty patients diagnosed as suffering from atypical virus pneumonia, only thirty-seven have been investigated for the presence of MG agglutinins. Of these thirty-seven patients only eleven have shown a fourfold or greater rise in titre of paired sera or a titre of 1/40 or more if single specimens were tested.

From these thirty-seven patients altogether seventy-five serum samples have been investigated. These seventy-five serum samples including those drawn from the twenty-six MG-negative patients as well as those samples which have been taken from the eleven MG-positive patients before and after the MG-positive phase of their illness, showed a distribution of MG-titres as recorded in Table 13. Not a single serum sample from the twenty-six MG-negative patients showed a titre exceeding 1/10, a fact which explains the predominance of the low titres in Table 13. Of the seventy-five serum samples taken from patients diagnosed as atypical virus pneumonia only nine samples or 12 % of the sera obtained from the eleven MG-positive patients showed a titre of 1/40 or higher. The highest MG-titre observed was 1/320.

Besides the seventy-five sera obtained from patients with atypical virus pneumonia we have also investigated 407 samples of serum from patients who had no signs of pneumonia and had been diagnosed as hepatitis or influenza, lymphocytic choriomeningitis, mumps, Q-fever or leptospirosis as the result of both clinical and laboratory tests. Table 14 presents the results of the MG agglutination reaction with the 482 sera, arranged according to diagnoses and titres.

Of the 274 hepatitis sera tested ninety-nine showed a titre of 1/10 or 1/20, and sixteen a titre of 1/40. Of the remaining 133 serum samples tested for MG agglutinins, not a single one had a titre above 1/20.

Both the incidence and the height of the MG agglutinins in cases of atypical virus pneumonia, so far diagnosed in Serbia, show fairly low levels when compared with the results reported from some other countries.

Table 13. *Streptococcus MG* titres of sera from patients with atypical virus pneumonia

	Titres of MG agglutinins							Total
	< 10	10	20	40	80	160	320	
No. of patients suffering from atypical virus pneumonia	40	12	14	6	2	0	1	75

Table 14. *Streptococcus MG* titres of sera from patients who had been diagnosed clinically as suffering from diseases other than atypical virus pneumonia

Clinical diagnosis	Titres of MG agglutinins					Total no. of sera
	< 10	10-20	40	80	160	
Atypical virus pneumonia	40	26	6	2	1	75
Hepatitis	159	99	16	0	0	274
Influenza, lymphocytic choriomeningitis, mumps, Q-fever and leptospirosis	99	34	0	0	0	133

Table 15. *Distribution of titres of sera from patients suffering from Q-fever compared with the titres of sera from patients with other diseases and from apparently healthy individuals when tested with Q-fever antigen.*

	Reciprocals of the titres								Total
	< 8	8-16	32	64	128	256	512	1024	
No. of sera from Q-fever patients	7	2	9	11	11	8	7	19	74
No. of sera from patients with infections other than Q-fever and from apparently healthy individuals	941	86	18	3	0	1	0	0	1049
Total no. of sera tested with the Q-fever antigen									1123

Q-fever

Table 15 presents the distribution of serum titres observed both in seventy-four samples of serum from definite cases of Q-fever and in those sera obtained from apparently healthy persons as well as from patients with infections other than Q-fever. Of the 1049 sera obtained from this latter group only four specimens showed a titre of 1/64 or higher. All four were single specimens from different cases. Two were from shepherds in charge of herds which had been proved by serological tests to be infected with Q-fever; both shepherds had been apparently healthy for the last 3 years. One was from a girl and one from an elderly woman, both of whom were members of the same family in which serologically proved acute Q-fever cases had occurred at the time when the sera were obtained. Whether these four examples represent recent inapparent infections or recovered cases with agglutinins persisting for some years cannot be determined with single samples of serum.

Table 16 records the titres found in the seventy-two serum samples from acute Q-fever cases grouped according to the number of days from the beginning of the disease that the serum was collected.

Only once have we seen a titre of 1/64 as early as the 6th day of the disease and in two cases titres of 1/512 or 1/1024 as late as the 90th day of the disease.

Table 17 records the results obtained on testing for Q-fever about 500 sera from various animal species.

Table 16. *Variation in titre of Q-fever antibody with the number of days from the onset of disease*

	Days from onset of disease				Total
	1-7	8-28	29-60	> 60	
No. of sera tested	8	35	22	7	72
Geometrical means of the titres found	1/4	1/154	1/355	1/211	1/123

Table 17. *The distribution of Q-fever antibodies in the sera of different animals*

Animals species	Reciprocals of the titres						Totals
	< 8	8	16	32	64	> 64	
Sheep	223	48	27	18	5	4	323
Lambs	11	0	0	0	0	0	11
Cows	157	7	0	0	0	1	165
Bulls	3	0	0	0	0	0	3
Calves	2	0	0	0	0	0	2
Goats	3	0	0	0	0	0	3
Dogs	1	1	0	0	0	0	2
No. of sera tested							509

Typhus fever

We have been able to diagnose forty-six cases of typhus fever during this period of 15 months. Altogether 381 samples of serum were tested with the epidemic typhus antigen. Of this number, sixty-four samples were obtained from the forty-six positive typhus patients.

Table 18 records the geometrical mean values of the titres observed in the sera of the typhus patients distributed according to the interval in days from the onset of the disease.

Table 19 shows the distribution of titres of the sera tested with the typhus antigen. Besides the sera obtained from typhus patients we have also tested as controls 317 specimens obtained from healthy persons or patients developing symptoms unrelated to typhus fever. In this control group of sera twenty-six samples gave a titre of 1/32 or higher. These twenty-six samples all belonged to individuals living in areas where typhus fever is endemic.

Until further experience of diagnosing the disease serologically has been obtained in countries such as ours, where typhus is still a common infection, it would seem to us to be advisable to test at least two samples of serum and to demonstrate a rise in titre with time before making a positive diagnosis and if this is impossible to adopt a minimum titre of 1/256. Only if supported by strongly suggestive epidemiological and clinical evidence and accompanied by a positive Weil-Felix reaction have we included single samples of serum with titres of only 1/64 or 1/128 in the group of positive typhus results.

The Weil-Felix reaction has not been performed as a routine in our laboratory.

Thirty-four of the typhus-positive sera have been tested both with epidemic and with murine antigens. Not one of the thirty-four sera gave a titre that was higher with the murine antigen than with the epidemic antigen. Only six sera showed equal titres with both antigens and the remaining twenty-eight sera gave a titre two to eightfold higher with the epidemic than with the murine antigen.

Table 18. *Variation in titre of typhus antibodies with the number of days from the onset of disease*

	Days of the disease				Undetermined	Total
	1-7	8-28	29-60	> 60		
Number of sera tested	0	37	14	12	1	64
Geometrical means of the titres found	—	1/595	1/222	1/90	1/1024	1/337

Table 19. *Typhus antibody titres of sera of persons diagnosed as suffering from the disease compared with the sera of normal persons and patients other than typhus*

	Reciprocals of titres										Total
	< 8	8-16	32	64	128	256	512	1024	2048	4096	
No. of sera of typhus patients	0	0	3	10	8	8	13	17	4	1	64
No. of sera of normal persons and of patients other than typhus	256	34	24		2	0	0	0	0	0	317

Lymphocytic choriomeningitis

Table 20 shows the distribution of titres both in the sera originating from patients suffering from lymphocytic choriomeningitis (seventeen samples) and those from patients infected with other agents (193 samples). These 193 samples were obtained from patients suffering from other varieties of meningitis or from some respiratory syndrome. Many of these patients were shown to be suffering from one of the following infections: mumps, leptospirosis, influenza, atypical virus pneumonia, Q-fever, etc. Only three of these 193 sera originating from three different patients had titres as high as 1/2 and not one had a titre higher than 1/2.

Besides the 210 sera recorded in Table 20 of which seventeen originated from cases of lymphocytic choriomeningitis and 193 from patients with other diseases, we have also investigated twelve sera which showed an equivocal result and, therefore, were not included in the material recorded in Table 20. These 12 sera originated from patients clinically suspected to be suffering from lymphocytic choriomeningitis infections. Two varieties of equivocal reactions have been observed. One when, in the routine test, a positive reaction occurred in a series of tubes but in none reached a two or three-plus fixation and usually showed one or more zones; the second type of equivocal reaction has been obtained with anti-complementary sera and with sera which fixed complement in the presence of normal guinea-pigs' spleen antigen.

Although for analysing our material a critical titre of 1/8 has been arbitrarily

adopted the data presented in Tables 20 and 21 suggest that a titre of 1/4 can safely be accepted as critical for lymphocytic choriomeningitis virus infection if supported by suggestive clinical evidence.

Table 20. *Serum titres of persons diagnosed as suffering from lymphocytic choriomeningitis compared with the serum titres of normal persons and patients suffering from other diseases*

	Reciprocals of titres							Total
	< 2	2	4	8	16	32	64	
No. of sera of lymphocytic choriomeningitis patients	3	0	2	5	2	4	1	17
No. of sera of patients other than lymphocytic choriomeningitis	190	3	0	0	0	0	0	193

Table 21. *Lymphocytic choriomeningitis titre variations with the number of days from the onset of disease*

	Days from onset of the disease			
	0-7	8-28	29-60	Total
No. of sera tested	2	10	5	17
Geometrical means of the titres	1/2	1/14	1/8	1/8.7

Table 22. *Mumps antibody titre variations with the number of days from the onset of the disease*

	Days of the disease				Total
	1-7	8-28	29-60	60	
No. of sera tested	13	31	7	1	52
Percentage of sera with titres 1/64 or higher	23	55	71	—	48
Geometrical means of the titres found	1/14	1/49	1/48	1/8	1/25

Table 23. *Mumps antibody titres of sera of persons diagnosed as suffering from the disease compared with sera of patients suffering from other diseases*

	Reciprocals of the titres							Total
	< 8	8-16	32	64	128	256	1024	
No. of sera of mumps patients	8	8	11	14	4	6	1	52
No. of sera of patients other than mumps	272	75	18	0	0	0	0	365

Mumps

In Table 5 the twenty-eight mumps patients are classified according to clinical symptoms.

Table 22 shows that between the 8th and 60th day of mumps infection the mean titres do not exceed 1/50. However, Table 23 shows that eighteen out of the 365 sera obtained from patients infected with agents other than mumps, showed a titre of 1/32. These two facts would seem to justify the recommendation that

sera from cases suspected of mumps infection should be tested in pairs to show titres rising with time. It may well be advisable to test each serum both with viral and with soluble antigen separately.

Infectious mononucleosis

The great majority of our positive results have been obtained from clinical cases having pronounced diphtheria-like angina.

Of the fourteen patients considered clinically to be typical cases of infectious mononucleosis, only ten gave positive Paul-Bunnell reactions. Thus, in our hands, the Paul-Bunnell reaction was positive in no more than 71 % of the infectious mononucleosis cases. It may be hoped that by sending earlier and more frequent blood samples, this percentage may be somewhat increased. No attempt has been made to differentiate listerellosis from our cases of infective mononucleosis.

Fig. 1. Variations in the titre of infectious mononucleosis antibodies with the number of days from the onset of the disease. x Serum samples showing lower titres than the previous sample or samples; o, serum samples showing peak titres or to be followed by samples with higher titres; ●, titres found in single serum samples.

Of the fifty blood samples drawn from patients with infectious mononucleosis, twenty-four were peak or pre-peak, nineteen were post-peak samples and seven were single specimens. Fig. 1 illustrates the distribution of the titres in these fifty specimens according to the day of the disease. The same Text-figure shows that, on the one hand, we have had examples of titres falling below 1/10 as early as the 11th and 17th day of the illness while, on the other hand, in one case the Paul-Bunnell level kept below 1/10 as late as the 23rd day to be followed by a rise which became fourfold 10 days later. The great individual variation observed in the time of appearance, increase or fall of the Paul-Bunnell reaction titres seems to demand that sera should be tested as early and as frequently as possible during the course of the illness.

Table 24 shows clearly that not one of the 266 sera obtained from patients with infections other than infectious mononucleosis has shown a Paul-Bunnell titre

higher than 1/10. We consider, therefore, that it is justifiable to take a titre of 1/20 as positive if proceeded or followed by a serum sample which is negative at a dilution of 1/10. Of the great number of sera from cases of meningitis tested with the Paul-Bunnell reaction, not one was positive.

Table 24. *Infectious mononucleosis antibody titres of sera of persons diagnosed as suffering from the disease compared with the sera of patients suffering from other diseases*

	Reciprocals of the titres					Total
	< 10	10	20	40	> 40	
No. of sera of infectious mononucleosis patients	10	1	6	10	23	50
No. of sera of patients other than infectious mononucleosis	248	18	0	0	0	266
Total no. of sera tested with the Paul-Bunnell reaction						316

Leptospirosis

Tables 25 and 26 present the results of testing 103 sera obtained from forty-one patients found to be infected with leptospirae. The distribution of the different types of leptospirae causing infection of the forty-one patients, diagnosed during the 15 months period was as follows: *L. sejroe* 18, *L. icterohaemorrhagiae* 4, *L. pomona* 18, and *L. mitis* 1.

Table 27 shows the results of testing 164 animal sera. In the animal as well as in the human sera a prevalence of both *L. pomona* and *L. sejroe* antibodies may be noted.

About 450 serum specimens originating mostly from patients diagnosed as retinitis, uveitis, etc., and grouped under the syndrome 'Miscellaneous' at the beginning of this report have been received from ophthalmology departments for testing systematically for leptospirosis. No cases of recent infection have been discovered. A certain number of sera with titres mostly around 1/300 indicated a previous contact with one or other of the leptospira strains. These results, although highly appreciated by the ophthalmologists, are not recorded in this report, because no suggestive evidence could be obtained, such as a significant rise or fall in the titres, that the patients were suffering from acute leptospiral infection.

Toxoplasmosis

As yet only about forty single samples of sera have been investigated with the complement-fixation reaction, using the toxoplasma antigen already described. Of the forty sera tested, two showed a positive result. The two patients and the titres found in their sera were the following:

Patient 1. A newborn infant with an acute infection whose serum showed a titre of 1/32.

Patient 2. An apparently healthy man, aged 29, showing a titre of 1/2 (+ + +) and 1/4 (±). This man is the father of a 3-month-old baby with pronounced hydrocephalus but a negative toxoplasma complement-fixation reaction.

Table 25. *Leptospiral antibody titres of sera of persons diagnosed as suffering from the disease compared with sera of patients suffering from other diseases*

	Reciprocals of the titres						Total
	< 300	300	1000	3000	10000	30000	
No. of sera of leptospirosis patients	13	17	45	17	10	1	103
No. of sera of apparently healthy persons and of patients other than leptospirosis	2897	80	0	0	0	0	2977

Table 26. *Variations in the titre of leptospiral antibodies with the number of days from the onset of the disease*

	Days of the disease					Total
	1-7	8-28	29-60	More than 60	Undetermined	
No. of the sera tested	5	44	26	24	4	103
Geometrical means of the titres	1/80	1/1140	1/1430	1/290	—	1/855

Table 27. *The distribution of leptospiral antibodies in different animals' sera*

Animal species	Reciprocals of the titres				Total
	Less than 30	30-100	300-1000	More than 1000	
Sheep	68	<i>L. sejroe</i> 1 <i>L. mitis</i> 1	<i>L. sejroe</i> 2	0	72
Cows	47	<i>L. pomona</i> 1 <i>L. sejroe</i> 10 <i>L. mitis</i> 3	<i>L. sejroe</i> 2	0	63
Horses	4	<i>L. pomona</i> 4 <i>L. sejroe</i> 1 <i>L. ictero.</i> 3	<i>L. pomona</i> 10 <i>L. ictero.</i> 1 <i>L. austral</i> 1	<i>L. pomona</i> 2	26
Dogs	1	<i>L. ictero.</i> 1	0	0	2
Mules	0	0	<i>L. pomona</i> 1	0	1
No. of sera tested of all species of animals					164

DISCUSSION

In calculating the geometric means, titres lower than 1/8 have been taken as 1/2, except for the leptospira titres which when lower than 1/30 have been taken as 1/10, which fact makes the mean titres somewhat lower than they should be.

Tests with the psittacosis and rickettsial-pox antigens were not performed as a daily routine, and the results obtained with these antigens will be reported in another paper.

The haemagglutination-inhibition tests for influenza and mumps performed with pairs of sera gave us results in all tests which confirmed those obtained with the complement-fixation technique.

Of the total of 4036 serum samples tested, more than 90 % originated from Serbia, and less than 10 % from Bosnia. The sera originating from Bosnia were

drawn mostly from apparently healthy persons and animals and were sent for testing for Q-fever, typhus and leptospirosis.

As stated earlier, out of the 3500 samples of human sera received, 638 gave positive tests. However, 1310 of these serum samples were drawn from patients grouped as suffering from hepatitis or miscellaneous diseases. Not one of these 1310 sera gave a positive result, nor were any of them drawn from patients suspected to be suffering from any of the virus infections which we are able to diagnose at the present time. Subtracting these 1310 samples from the total of 3500, we are left with 2190 serum samples, originating from patients who have been grouped under the following syndromes: pyrexia of unknown origin, pneumonia, meningitis, grippe, status typhosus, and of these 638 samples gave positive results (see Table 28).

Table 28. *Distribution of positive results of serological tests*

	Total	Total with diagnosis of hepatitis or miscellaneous	Total all other diseases	Total positive	Percentage of the positives calculated on the basis of	
					All diseases	All syndromes except hepatitis and mis- cellaneous
Sera	3500	1310	2190	638	18.2	29.2
Patients	2430	c. 1310	c. 1120	334	13.7	c. 30

We conclude from the figures in Table 28 that about 30 % of the patients, whose sera have been submitted with a more or less justified suspicion of virus infection have been diagnosed as positive in our laboratory.

Of the 365 sera drawn from healthy persons and patients suffering from infections other than mumps, not one has revealed a titre higher than 1/32. The adoption of a critical titre of 1/64 would probably exclude all cases which were not actual infections with mumps virus, but at the same time would miss a certain number of acute mumps infections showing titres of 1/32 or less. For these and similar reasons, stress is laid on testing paired samples of sera when mumps infection is suspected.

The infections of mumps meningitis, lymphocytic choriomeningitis and toxoplasmosis which were proved serologically are the first cases to be reported from Yugoslavia.

SUMMARY

An analysis is given of the serological tests performed over a period of 15 months with viral, rickettsial and leptospiral antigens. The analysed material is made up of about 9400 tests performed on 4036 samples of serum obtained from 2430 patients and 536 animals.

The incidence of the various diseases, the distribution of the positive results according to diseases, and the height of the specific titres, as well as the height of the residual titres found in the material, are discussed and analysed in detail.

The procedure for the preliminary screening of the material, as well as the methods used in performing the different serological tests, are described and discussed.

A few examples of possible double infections are quoted, namely atypical virus pneumonia with influenza, and influenza A with B.

An analysis of the results of 1723 tests performed with influenza antigens on 909 samples of sera is presented.

The results obtained from testing about 1000 sera for cold agglutinins and 482 sera for MG agglutinins are discussed in detail.

Some cases of liver affections showed a marked rise in titre both of cold agglutinins and of MG agglutinins.

The serum samples, numbering about 1050, drawn from normal persons or patients suffering from infections other than Q-fever, all gave titres lower than 1/64 when tested with Q-fever antigen, except the sera of four persons who were probably cases of recent inapparent infection.

The geometrical mean of the titres found in sera drawn from acute Q-fever patients between the 29th and 60th days of their illness was 1/355. Of the 500 sera from various animals, 100 from sheep and eight from cows had titres of 1/8 to 1/64 against the Q-fever antigen.

Thirty-four sera have been positive when tested with the soluble antigen of both the epidemic and the murine types of typhus but no serum has given a higher titre with the murine type antigen than with the epidemic type antigen. Of the thirty-four sera tested with both antigens the titres obtained with the epidemic antigen were higher than with the murine antigen in 28.

The results obtained with 417 sera tested with the mumps antigen, and with the 222 sera tested with the lymphocytic choriomeningitis antigen are reported and discussed in detail.

Great individual variation has been observed in the time of appearance and rise and fall of the Paul-Bunell titres. Consequently, it is advised that early and frequent blood samples should be obtained from patients who are suspected to be suffering from infectious mononucleosis. On the basis of the results of 316 Paul-Bunnell tests it is suggested that a titre of 1/20, if preceded or followed by a negative serum sample, should be taken as conclusive evidence of infectious mononucleosis.

3080 human and 164 animal sera have been investigated for the presence of antibodies to various types of leptospirae. In both human and animal sera antibodies have been found most frequently against *L. sejroe* and *L. pomona*.

The results of a few tests performed with toxoplasma antigen are mentioned briefly.

We wish to express our gratitude to all our colleagues in Belgrade and other parts of the country who have provided us with information and additional samples of blood when requested, in particular Prof. K. Todorowich of the Infectious Diseases Hospital, Belgrade, and Drs Lj. Vuksich, M. Morelj, B. Arsich, D. Mehl and R. Papo of the Army Medical School, Belgrade.

We wish to thank Dr R. Djorich of the Infectious Diseases Hospital, Belgrade, for the supply of material for testing for atypical virus pneumonia and aseptic meningitis, and Dr Ž. Perishich for help with our cases of infectious mononucleosis. Mrs Martha Milivojevich gave valuable assistance with the complement-fixation tests and with the compilation of material for this report.

REFERENCES

- BARRETT, A. M. (1941). *J. Hyg., Camb.* **41**, 330.
HOYLE, L. (1948). *Mon. Bull. Minist. Hlth. Lab. Serv.* **7**, 114.
REPORT (1953). *W.H.O. Tech. Rep. Ser.* **64**, 24.
RICHARDSON, G. M. (1941). *Lancet*, **2**, 696.
SCHÜFFNER, W. & MOCHTAR, A. (1927). *Proc. Acad. Sci. Amst.* **30**, 25.
SMADDEL, J. E., BAIRD, R. D. & WALL, M. J. (1939). *Proc. Soc. exp. Biol., N.Y.*, **40**, 71.
TERZIN, A. L. (1950). *Srpski Arh.* vi, 386.
TERZIN, A. L. (1952). *Srpski Arh.* iv, 241.
THOMAS, L., MIRICK, G. S., CURNEN, E. C., ZIEGLER, J. E. & HORSFALL, F. L. (1945). *J. clin. Invest.* **24**, 227.

(*MS. received for publication 4. VIII. 53*)