virus that affect both central nervous system and muscle have shown that suspensions of legs are either less virulent or about as virulent as suspensions of brains. This conforms with the signs of infection, with the histologic findings, and with the degree of creatinuria associated with the two types of disease.

Discussion. Whether or not these observations have application in clinical medicine is unknown. Creatinuria follows paralytic poliomyelitis[11-13] but has been assumed to be a sequel of neurone destruction. Since there is some evidence that muscle degeneration may occur in the initial stages of the disease,[14] the subject should be re-examined. Hassin described a case of poliomyelitis of the Landry type in which the lesions were restricted to the muscles.[15] Clawson is reported to have found no muscle lesions in a series of 22 cases of acute poliomyelitis[16] but Dr. Kornel Terplan, Buffalo General Hospital Laboratory, provided us with histologic preparations from a case of fatal bulbar poliomyelitis in which extensive muscle lesions similar to those which occur in suckling mice were seen.

The infectivity of the muscles is consistent with the morphologic changes and reaffirms the peripheral nature of the paralysis in the mice. It is interesting that the higher titer of the muscle preparations makes it possible to induce paralysis in mice of an age-group that is resistant to infection with brain suspension. While these findings modify, they also confirm the original observation of the remarkable natural immunity of mice and hamsters following weaning. This is further supported by failure to adapt the T.T. strain to weaned mice by alternate passage through susceptible animals.

Summary. Infection of unweaned mice with certain strains of "Coxsackie virus" is followed by loss of muscle potassium and creatinine and by creatinuria.

The muscles of paralyzed mice are highly infectious.

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17267. The Role of Complement in the Lysis of Leucocytes by Tuberculoprotein.*

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(Introduced by J. H. Mueller.)

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The nature of the mechanism underlying delayed or tuberculin-type hypersensitivity has long interested students of immunology. Whereas many workers have attempted to demonstrate an antibody in tuberculin allergy which was similar in properties and mode of action to antibodies known to be responsible for anaphylactic or Arthus type of hypersensitivity, the preponderance of such studies has led to the assumption that cells, rather than serum antibodies, were the mediators of tuberculin hypersensitivity. Holst[1] first noted

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decreased phagocytic activity and loss of differentiation between the nucleus and cytoplasm when tuberculin was added to leucocytes derived from tuberculous animals but no deviation from normal when horse serum was added to leucocytes from guinea pigs sensitized to horse serum. Similarly Stewart *et al.*[2] observed that tuberculin killed leucocytes derived from the tuberculous guinea pig while the leucocytes of a guinea pig sensitized to crystalline egg white were not affected by the addition of that specific antigen.[3] These and other studies[4-9] have distinguished this cytotoxic role of antigen on cells of tuberculin-type allergic animals from the harmless response of cells to antigen in the anaphylactic forms of hypersensitivity. In the latter instance, serum antibody is felt to be the crucial factor in the hypersensitivity manifestations. The failure to transfer passively tuberculin sensitivity by means of the serum of a sensitized animal and the successful transfer of such sensitivity with cells of the tuberculous guinea pig by Chase[10] and others[11-13] have given further support to the concept that cells, rather than serum antibody, were the prime mediators of tuberculin type hypersensitivity.

The studies of Favour,[14] using a one-hour period of *in vitro* observation, have provided a simpler method of determining the cytotoxicity of tuberculin and subsequent investigation in this laboratory has aimed at elucidating the mechanism of tuberculin allergy by this *in vitro* technic. Earlier work[15] with white cells of tuberculous humans indicated that tuberculin was specifically toxic to these cells. It was further assumed that complement was essential for such cytolysis since no tuberculin cytolysis was observed if the diluting serum was heated at 56°C for 30 minutes.

A more recent report from this laboratory,[16] however, has shown that it is unnecessary to use "sensitized tuberculous cells"§ to demonstrate short term tuberculin cytolysis. *In vitro* lysis of leucocytes by tuberculo-protein has been found to be dependent on the presence of tuberculous plasma (or serum). Further studies[17] now make it apparent that the active component of tuberculous plasma is a heat labile globulin. This finding of an antibody-like factor in tuberculous plasma (or serum) which is responsible for the cytotoxic action of tuberculin on tuberculous as well as on normal leucocytes‖ prompted a reinvestigation of the role of complement in this reaction. Because of the heat lability of the plasma factor, it was necessary to resort to other methods of removing complement completely from tuberculous plasma (or serum).

Experimental. As in previous experiments,[16-17]§ white blood cells from a normal, healthy tuberculin-negative subject were separated, washed 3 times in saline and used as the target cell for determining tuberculin

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§ White cells from a patient having active tuberculosis.

17 Miller, J. M., Favour, C. B., Wilson, B. A., and Umbarger, M. A., PROC. SOC. EXP. BIOL. AND MED., 1949, **71**, 287.

‖ Cells from a normal, healthy, tuberculin-negative subject.

cytolysis, thus leaving the tuberculin antigen, the tuberculous plasma factor and complement as the 3 vital determinants of eventual cell breakdown. Plasma (or serum) was obtained from patients hospitalized for active pulmonary tuberculosis whose plasma had previously been shown to contain the active plasma factor. Plasma from normal, healthy tuberculin-negative donors was used as a control. In order to remove complement completely from this plasma without inactivating the heat-labile plasma factor essential for cell lysis, two separate antigen-antibody systems were used. In the first instance, pneumococcal type III polysaccharide and antipneumococcal type III rabbit antiserum (previously found to produce a precipitate with antigen diluted 1:2,000,000)¶ were added to tuberculous plasma and normal plasma in such dilution as to give a precipitate in the zone of equivalence for this antigen-antibody system and thus fix complement. This mixture was incubated at 37°C in a water bath for 2 hours and then in the ice box at 10°C for 2 days. As a second complement-fixing antigen-antibody system, crystalline bovine albumin and anti-bovine albumin rabbit serum (previously found to produce a precipitate with antigen diluted 1:800,000)¶ was similarly added to tuberculous and normal plasma so as to yield precipitate in the zone of equivalence for that system. Complement titers were determined on the untreated tuberculous and normal plasma as well as on such plasma to which had been added the pneumococcal polysaccharide and bovine albumin systems in order to confirm the complete absence of complement from the latter two systems.

In estimating the amount of complement activity present in individual untreated sera, a modification of the method proposed by Mayer *et al.*[18] was employed. Using 0.5 ml amounts of 4% sheep cell suspensions, sensitized with four units of high titer amboceptor, as the indicator system, the amount of serum necessary to produce 50% hemolysis was determined. The final titer was calculated from the equation

$$C = \frac{D}{V} \times 2.5$$

where C = the number of 50% hemolytic units of complement present per ml of undiluted serum, D = the reciprocal of the dilution of serum used, V = the volume of this dilution necessary for 50% hemolysis, and 2.5 is a factor used in this laboratory to make the results referable to those of our determinations which use a system employing only 0.2 ml of sensitized sheep cells. By this method normal human sera usually have complement titers in the vicinity of 300 units per ml. Plasma may show slightly lower values because of the anticoagulant used. All dilutions were made with a veronal buffered saline containing optimal quantities of calcium and magnesium ions.[19]

In those sera from which complement had been removed, a titration procedure designed to detect complement sufficient to produce 50% hemolysis was obviously not applicable. It was necessary to detect minimal hemolysis. Accordingly, to 0.2 ml suspensions of sensitized sheep cells were added the test sera in dilutions just sufficient to abolish non-specific hemolysis and/or anticomplementary factors. The final volume was 0.8 ml. After incubation for 40 minutes at 37°C the cells were centrifuged and the supernatant examined for evidence of hemolysis. In no instance, with either of the complement-fixing antigen-antibody systems used, was there any demonstrable complement activity remaining in the tuberculous or normal sera.

In order to demonstrate that the cytolysis promoting capacity of such plasma could be restored by the readdition of complement, freshly drawn type AB serum from a tuberculin-negative subject was titered for complement and added to the "decomplemented" plasma in separate tubes. As the tuberculin antigen in all experiments, Old Tuberculin,

¶ Kindly supplied by M. H. Kaplan of the Department of Bacteriology, Harvard Medical School.

[18] Mayer, M. M., Eaton, B. B., and Heidelberger, M., *J. Immunol.*, 1946, **53**, 31.

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TABLE I.

Effect of Complement on *in vitro* Lysis of Normal Leucocytes by Tuberculin in Presence of Normal and Tuberculous Plasma.

	Complement 50% hemolytic units per ml																
Normal cells		0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	
Tuberculous plasma	217	0.3	0.3	0.3	0.3												
Tuberculous plasma "decomplemented"*	0					0.6	0.6	0.6	0.6								
Normal plasma	234									0.3	0.3	0.3	0.3				
Normal plasma "decomplemented"*	0													0.6	0.6	0.6	0.6
Fresh type AB serum	214			0.1	0.1			0.1	0.1			0.1	0.1			0.1	0.1
Tuberculin antigen		0.1		0.1		0.1		0.1		0.1		0.1		0.1		0.1	
Saline		0.1	0.2		0.1	0.1	0.2		0.1	0.1	0.2		0.1	0.1	0.2		0.1
Total WBC 5 min.		9,650	9,330	9,770	9,330	6,270	6,330	6,130	6,260	10,110	10,190	7,725	7,320	5,400	5,480	4,980	6,120
60 min.		7,670	9,260	7,790	9,200	6,300	6,310	4,930	6,330	10,060	10,280	7,650	7,270	5,330	5,470	5,000	6,080
% Decrement		—20.5	—0.7	—20.2	—1.4	+0.5	—0.5	—19.5	+0.7	—0.5	+0.7	—0.8	—0.7	—1.2	—0.5	+0.2	—0.5

* To remove complement from 2 ml of plasma, 1 ml of a 1:1000 solution of crystalline bovine albumin and 1 ml of anti-bovine albumin rabbit serum was added; thus, final dilution of plasma was 1:2.

prepared as described earlier,[20] was used. From previous experiments, it was found best to use cells and plasma from homologous blood groups.

The methods of preparing and washing white cell concentrates, diluting these cells in the various plasma (or sera) and cell counting to estimate cytolysis were the same as described previously.[16,21]

Results. The results of a typical experiment are recorded in Table I. In this instance, complement was removed by means of the crystalline bovine albumin system but identical findings were observed with the pneumococcal polysaccharide antigen-antibody system.

1. As reported earlier,[16] a portion of thoroughly washed white blood cells from a healthy, tuberculin negative subject undergoes significant cytolysis when incubated for one hour at 37°C with freshly drawn tuberculous plasma and tuberculin antigen. No such cytolysis occurs if the plasma of a tuberculous patient is replaced by the plasma of a tuberculin-negative subject.

2. Removal of all complement from tuberculous plasma by means of an unrelated complement-fixing antigen-antibody system results in the loss of the ability of such plasma to affect cytolysis of white blood cells. The successful removal of all complement by this technic has been confirmed by the sheep cell hemolysis method described above. It can be noted that 0.6 ml of "decomplemented" plasma was used instead of the 0.3 ml used for fresh plasma. This was done since the complement fixing procedure resulted in a 1:2 dilution of the original plasma.

3. The addition to the system of active complement in the form of freshly drawn type AB tuberculin-negative serum restored completely the capacity of "decomplemented" tuberculous plasma to cause white cell destruction in the presence of Old Tuberculin.

4. Plasma from a tuberculin negative subject continued to have no effect on white blood cells even after the addition of fresh complement.

5. The addition of further complement of known titer to this system in the form of freshly drawn AB serum from a tuberculin-negative donor does not further enhance the tuberculin cytolysis of this system. It may be assumed, therefore, that the tuberculous plasma already has adequate amounts of complement.

Discussion. The present report further elucidates the mechanism of the *in vitro* cytolysis of human white blood cells by tuberculoprotein. Contrary to the original hypothesis in this laboratory that tuberculin cytolysis was a direct toxic effect of tuberculin on the tuberculous "sensitized" cell, a more recent report[16] has shown that even normal white cells of a tuberculin negative donor can be lysed by tuberculin provided a factor in tuberculous plasma is also present. Various properties[17] of this factor suggest that it is a globulin. This work further characterizes such *in vitro* cytolysis as a manifestation of an antigen-antibody inter-action. It now appears evident that complement too is an essential component of this system.

The question of most significance in these studies is whether or not such *in vitro* short term tuberculin cytolysis, dependent as it is on antigen, antibody and complement, may be taken to be a valid analogue of *in vivo* cutaneous tuberculin hypersensitivity. If this be so, then the dependence of the antigen-antibody reaction upon complement has significance in indicating the role of the lytic action of this substance in this type of hypersensitivity. The successful passive transfer of tuberculin hypersensitivity in guinea pigs by Chase,[10] using living cells, allows the possibility that the hypersensitivity developed in the recipient animal may be due primarily to elaboration by the cells of antibody which is identical in properties to the plasma factor demonstrated in this laboratory.

Summary. The essential role of complement in the *in vitro* cytolysis of human white blood cells by tuberculin has been demonstrated, using two different systems of complement removal. In this way, the mechanism

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of *in vitro* tuberculin hypersensitivity has been further elucidated.

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17268. Alloxan Subdiabetes in Rabbits Detected by Modification of Glucose Tolerance by Adrenal Cortex Extract.

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The subdiabetic state may be defined as a phase of pancreatic diabetes characterized by subtle metabolic disorders; these disorders, in contrast to overt diabetes, are unaccompanied by hyperglycemia or glycosuria, and, in contrast to latent diabetes, are not accompanied by impaired glucose tolerance as determined by conventional methods. The existence of such a state, which might or might not be a prediabetic state, has been suspected by many students of human diabetes mellitus.

Although latent diabetes has been demonstrated in various animals,[1-3] subdiabetes and prediabetes have only been demonstrated by retrospective analysis of the records of mothers of overweight babies[4] and of the records of partially pancreatectomized animals.[5,6] The present studies were undertaken in an attempt to produce experimental subdiabetes, and, by exploiting the adrenal cortex-insulin antagonism,[7] to devise a method for its detection.

Methods and Materials. Mongrel and white rabbits weighing 2.5-3.6 kg were used. Their diet consisted of oats, hay, carrots, cabbage, lettuce, and water *ad lib.* The animals were starved for 18-20 hours prior to each experimental procedure, including the alloxan injections. Sugar was determined on ear vein blood by the method of Hagedorn and Jensen;[8] no anticoagulant was used, and the precipitated samples were all permitted to stand, without fluoride, until the end of the third hour, when the determinations were made. Urinary sugar was not followed.

Glucose tolerance was tested by the intravenous injection, during about one minute, of 3 g of glucose in 50% solution, regardless of weight; this was followed by 2-3 cc of physiologic saline. Fasting, 1, 2, and 3 hour blood samples were drawn. The effect of adrenal cortical hormones on the glucose tolerance was studied by injecting aqueous adrenal cortex extract (Upjohn) intramuscularly, ½ cc/100 g body weight, one half hour before the glucose injection; the entire procedure will be called the *A. C. Tolerance Test.* It should be noted that the fasting blood sugar specimens in all A. C. tolerance tests were drawn one half hour after the injection of adrenal cortex extract (A.C.).

One day or more after the completion of preliminary tolerance tests, alloxan monohydrate (Eastman) was rapidly injected into the ear veins of fasted rabbits in 2.5% solution; further tolerance tests were performed, and subsequent doses of alloxan given, if indi-

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