

In a second type of experiment, the mold was grown at room temperature on cultures of tubercle bacilli. The controls were cultures of tubercle bacilli of the

TABLE 1

No.	Date	Cultures	Guinea pigs
41	3/21/44	---	living 7/21/44
58	3/21/44	---	living 7/21/44
control	3/21/44	+++	died at 53 days (Tbc at autopsy)
63	4/7/44	---	died at 99 days (Tbc at autopsy)
control	4/7/44	+++	died at 43 days (Tbc at autopsy)

same strain and were kept under the same conditions. At the end of two weeks, suspensions were made of the controls and of the mold and tubercle bacilli tubes by grinding with saline solution in a mortar. Cultures, smears and guinea pig injections were done after the suspensions had stood for 24 hours as in the previous experiments. Eleven such experiments have been done. In no instance have tubercle bacilli grown in the cultures of suspensions of mold and tubercle bacilli while the control cultures have all grown rapidly. As in the previous experiments, acid-fast organisms were found in all the smears. Ten of the experimental guinea pigs are alive and well; all these have negative tuberculin reactions. One animal died of an acute enteritis on the 88th day and showed no tuberculosis. The results of two such experiments are shown in Table 2.

TABLE 2

No.	Date	Cultures	Guinea pigs
14	2/26/44	---	living 7/21/44
control	2/26/44	+++	dead at 41 days
15	3/3/44	---	living 7/21/44
control	3/3/44	+++	dead at 84 days

Effect of the mold on Tuberculin and P.P.D.: The mold grew well on tuberculin in dilutions as high as 1:10,000 in saline solution. It also grew on dilutions in saline solution of P.P.D. (Purified Protein Derivative of Tuberculin), in concentrations of 0.05 mgm and 0.0002 mgm P.P.D. per ml. No growth occurred on saline controls. Solutions of tuberculin and P.P.D. upon which the mold had grown no longer produced positive skin reactions in guinea pigs which had been previously injected with tubercle bacilli, although control solutions of tuberculin and P.P.D. made in the same manner and left at room temperature for the same length of time produced typical skin reactions in the same animals.

Suspensions of the mold, prepared as described above, inactivated 1:100 tuberculin in two hours when left either at room temperature or at 37° C. Supernatant fluid obtained after centrifugation of the mold

suspension also inactivated 1:100 tuberculin. When the supernatant fluid was passed through a bacterial filter (E. K. Seitz), the filtrate did not inactivate tuberculin.

Experiments using fluid media, similar to that used for the production of penicillin, upon which the mold had been grown for 8 to 15 days, have shown no effect on tubercle bacilli or on tuberculin. *Staphylococcus aureus* grew on solid media upon which the mold had been grown and had been removed. Therefore it is believed that the substance produced by this mold is not similar to penicillin.

Experiments using extracts of the mold and of the supernatant fluid are in progress at the present time.

In 1910 Vaudremer³ observed that tuberculin, when added to filtered extracts of molds such as *Aspergillus fumigatus* and *Penicillium glaucum*, loses its activity. Furthermore, he observed that tubercle bacilli were modified by maceration in extracts of these fungi. Machado⁴ noticed that tubercle bacilli lost their acid fastness after three months' incubation in a culture of an unidentified mold. Smith and Emmart⁵ have shown that aqueous solutions of ether extracts of Raulin-Thom culture media of *Penicillium notatum* exhibited *in vitro* at certain concentrations, marked bacteriostatic activity against tubercle bacilli, although other preparations had little or no activity.

Conclusions: Suspensions of a mold which probably belongs to the *Penicillium* group inhibit the growth of tubercle bacilli. Suspensions of this mold inactivate tuberculin in two hours. Filtrates of these suspensions have no effect on tuberculin.

D. K. MILLER

ALBERT C. REKATE

IODINATION OF INSULIN

In an earlier report it was pointed out that the presence of a sufficient concentration of phosphate buffer salts at moderately elevated temperatures greatly increases the rate at which tyrosine reacts with certain oxidants.¹ This characteristic increased activity was found to be associated with the phenolic hydroxyl group. In view of the importance which has been attributed to this group, in regard to the physiological activity of a number of hormones, enzymes and proteins of disease this activity has been employed in correlating physiological and chemical activity and has been applied as a means of readily iodinating proteins or introducing other groups.

³ A. Vaudremer, *Ann. Inst. Pasteur*, 24: 189, 1910; "Le Bacille Tuberculeux," Les Presses Universitaires, Paris, 1927.

⁴ A. Machado, *Compt. rend. Soc. biol.*, 96: 484, 1927.

⁵ M. I. Smith and E. W. Emmart, *Pub. Health Rep.*, 59: 417, 1944.

¹ D. E. Bowman, *Jour. Biol. Chem.*, 141: 877, 1941.

Insulin was employed in the earlier phases of this study, since it contains considerably more tyrosine than do most proteins, its biological activity depending in part upon the phenolic hydroxyl of this amino acid. The object of the present preliminary note is to briefly describe some of the findings regarding relative reaction rates and solubilities associated with the iodination of insulin and other proteins. Other phases of the study will be described elsewhere.

A photoelectric photometer was used in observing relative iodination rates and degrees of turbidity. In some instances described here iodine was added to the experimental material and the depth of color observed (in the presence of starch) before phosphate buffer was added. The rate of iodine utilization was then followed to the point of complete decoloration. In other cases the relative turbidity resulting from the presence of the buffer and starch in the experimental solution was first noted and then the initial degree of color and the rate of decolora-

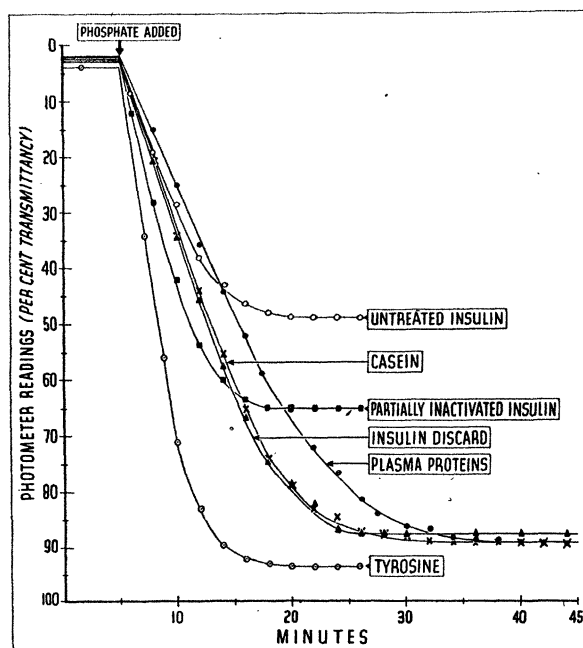


Fig. 1

tion were observed following the addition of iodine. The 0.001 N iodine used in these observations was freshly prepared from potassium iodate and potassium iodide in the presence of a moderate excess of hydrochloric acid. The starch solution consisted of 0.2 per cent. soluble starch according to Lintner. The solution was prepared by gently boiling for five minutes before diluting to volume. In some cases the temperature of the reaction medium was maintained at 38° C., while in other cases it was 50° C. The phosphate buffer solution consisted of a 0.8 M solution prepared according to Sorensen.

Employing 3 cc of an aqueous solution containing 0.1 mg of tyrosine with 3 cc of the starch, and 2 cc each of the iodine and the buffer solution (pH 5.9) it was found that a constant starch-iodine color remained before the phosphate was added. The rapid utilization of the iodine at 38° following the addition of the buffer is represented in Fig. 1. From the same figure the rapid reaction of 2 mg of amorphous insulin² under similar conditions is apparent. It may be observed that the iodinated insulin is less soluble under these conditions, giving the solution a decided turbidity.

On the other hand, it will be seen that after heating some of the same insulin in acid and alcohol the iodinated form was decidedly more soluble than the untreated insulin but still considerably less than inert proteins such as casein or plasma proteins. This treatment of the insulin was carried to the point at which the biological activity was decreased to about 50 per cent. of the original.

It is of interest to note that the curve representing the iodination of insulin discard, the last fraction which is discarded in the commercial preparation of insulin, resembles those of the inert proteins.

The reaction with crystalline zinc insulin resembles that of the untreated amorphous insulin. Protamine zinc insulin was also used, although a higher pH was employed in this case. Relative differences in the rates of iodination of insulin and the inert proteins are most apparent when smaller quantities of material are used and excess potassium iodide is employed in the preparation of the iodine solution.

By adding the iodine after the phosphate salts it is possible to more specifically estimate the change in turbidity accompanying the iodination. From Table 1 it will be observed that iodination amplifies the

TABLE 1

Amount of insulin	Per cent. transmittancy	
	Before iodination	After iodination
40 units	10.0	96.5
32 "	8.0	78.7
24 "	4.8	51.5
16 "	4.0	34.0

insolubility of the insulin under the conditions employed. In this series the original procedure was also altered by increasing the temperature to 50° C. and adjusting the sensitivity of the photometer in such a way as to narrow the range, approximately doubling all the readings. It may be observed that a reasonably close correlation exists between the concentration of the insulin and the turbidity following the iodination. In this latter series commercial insulin containing 0.2 per cent. phenol was used as

² We are indebted to Eli Lilly and Company for the various insulin preparations and the insulin discard.

the source of material. It was found that the added phenol could be completely extracted with ether without interfering with the subsequent insulin reaction. Deviation between concentration and turbidity before iodination was especially observed with the partially inactivated insulin. The characteristics of the iodination curves obtained in this manner were relatively constant and reproducible.

The iodination of insulin or other proteins also proceeds rapidly in the absence of starch. However, uniformity of results and the stability of the suspension of iodinated proteins were found to be improved in the presence of starch prepared as described.

DONALD E. BOWMAN

DEPARTMENT OF BIOCHEMISTRY

AND PHARMACOLOGY,

INDIANA UNIVERSITY SCHOOL OF MEDICINE,

INDIANAPOLIS

TRACER STUDIES WITH RADIOACTIVE SODIUM IN PATIENTS WITH PERIPHERAL VASCULAR DISEASE^{1, 2}

IN patients suffering from peripheral vascular diseases, the circulation time, volume of blood circulating in the extremities, and relation between plasma and extravascular fluid in the extremities may be altered. A study of these should be of diagnostic and prognostic value. The employment of radioactive sodium as a tracer substance in the blood is well suited to this purpose. It is easily prepared in the cyclotron, has a rather short half life (14.8 hours) and emits radiations which are readily detected. The injection of a few cu cm of normal saline into the blood stream can have no physiological effect, if the accompanying dose of radiation is insignificant. In the experiments here reported, this was the case.

The radioactive isotope is prepared by bombarding sodium metaborate in the cyclotron with deuterons. The metaborate containing the active atoms is dissolved in water, acidified with hydrochloric acid, and treated with methyl alcohol. The resulting methyl borate and water are driven off by heating, leaving sodium chloride. This is prepared in sterile solution in the desired concentration for injection.

When this material is injected into a vein it is carried throughout the vascular system, and the time required for it to reach a foot can be determined by placing a Geiger-Müller counting tube at the extremity and timing the interval between injection and audible registration by the counter. Since there is constant interchange of sodium between plasma and extravascular fluid, the amount of radio-sodium in the foot

will increase until equilibrium is established between these two. The manner in which this equilibrium is built up depends on the volume of blood traversing the region per unit time, and on the relation between intra- and extra-vascular space; these are related to the normal or diseased condition of the foot. By following the counting rate during a period after the administration of the isotope, information can be gained regarding this build-up.

The experimental procedure is as follows: The syringe containing 60-200 micro-curies of radio-sodium in 3-10 cu cm of saline is held close to the site of the injection, the Geiger-Müller tube, in a lead housing with a thin aluminum window, is placed against the sole of the patient's foot, and the scaling device on the counter adjusted so that 1 or 2 clicks are heard each 5 seconds. The injection and the electric stop-clock are started simultaneously, and counts recorded every 5 seconds, the time of the end of the injection also being noted. When the material reaches the foot, the number of clicks per 5 seconds increases definitely and sharply; the 5-second counting is, however, carried on for about a minute longer, to be sure that no erratic background has been mistaken for the arrival of the material. After this, the counting is carried on minute by minute as the build-up proceeds. After 10 or 15 minutes the counter is shifted to the other foot. Subsequently measurements are made at various regions of the legs, hands, etc., with frequent return to the feet.

Circulation times, arm to foot, have been determined in 35 individuals; the average value was 45 seconds, but the range was considerable. The highest was 90 seconds, in an elderly diabetic, arteriosclerotic woman who was fibrillating at the time of the test. The lowest was 15 seconds, in a young man in a highly nervous state, with a pulse of 100, suffering from scleroderma. In ten individuals considered to be normal as far as circulation was concerned, eight fell between 45 and 55 seconds; the other two were 60 seconds. Five cases of thromboangitis obliterans were all below 35 seconds. Seven arteriosclerotics showed a range between 30 and 80 seconds; it may be possible later to correlate circulation time with stage of disease. Four cases with ulceration and inflammation (three diabetics) showed times below 35 seconds.³

Probably more promising than circulation time as a clinical aid is the curve of equilibrium build-up. Fig. 1 shows data for the first 45 minutes for six normal individuals, five men and one woman, whose weights varied from 50 to 75 kg. (No adjustment is

¹ A preliminary report.

² From the Departments of Radiological Research and Surgery, College of Physicians and Surgeons, Columbia University, with the aid of a grant from the Lilla Babbit Hyde Foundation.

³ Hubbard, Preston and Ross have employed radio-sodium in a somewhat similar manner to determine circulation time in infants, although the present authors were unaware of this when they commenced their study. See *Jour. Clinical Investigation*, 21: 613, 1942.