STUDIES ON INSULIN.*

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This laboratory has prepared large amounts of insulin for use in the Potter Metabolic Clinic during the past 4 years. As opportunity afforded, various observations concerning the properties of insulin have been made. Our findings have been reported only in a preliminary manner because we believed that a more thorough study should precede publication. In this paper we report a method for preparing insulin, methods of purification, observations on the effects of various chemical reagents upon insulin, and an extended study of the labile sulfur of insulin. We also comment upon the chemical properties of insulin.

Preparation of Insulin.

The fresh pancreas of beeves and hogs are trimmed of fat and extraneous matter. They are kept on ice until a quantity has accumulated sufficient for a "run," about 30 pounds. The chilled glands are then ground to a pasty consistency in a Buffalo chopper. 20 pounds of this meat are placed in a rotary mixer together with 1 gallon of water and 100 cc. of concentrated sulfuric acid. After the mixer has run for 15 minutes, 5 gallons of 95 per cent alcohol (ethyl denatured with 10 per cent methyl) are added. The mixing is then continued to a total of 1 hour. The juice is pressed from this mixture by means of a heavy power press. At this point the concentration of alcohol should be 63 to 65 per cent and, if it is too low, enough 95 per cent alcohol is added to attain this concentration. The hydrogen ion concentration is about

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pH 2.5 to 3.0. The solution is next filtered. About 50 cc. of sodium hydroxide (sp. gr. of 1.50) are added to each 10 gallons of filtrate. It is important to make this partial neutralization rather exact in order to secure rapid filtration from the fat later in the process. The extract is concentrated *in vacuo* to about $\frac{1}{10}$ its volume. The temperature of this residue is about 45°C. upon removal from the water bath which is regulated to maintain a temperature of 80°C. The concentrate is filtered immediately through double folded filter papers. The filter papers containing the dark brown, fatty precipitate are extracted with enough water to cover and filtered the next day. The active material thus recovered is worth the labor involved. This filtrate is added to the main extract.

The clear aqueous filtrate is saturated by adding 22 per cent of commercial sodium chloride, which throws out a dark precipitate containing the active principle. After standing $\frac{1}{2}$ hour, the underlying solution is syphoned off. The salt precipitate is dissolved in the original volume of water and without changing the reaction again saturated with sodium chloride. The precipitate containing the active material rises to the surface and the salt water is again syphoned off and discarded. The second saturation with sodium chloride seems to remove most of the contaminating, inactive protein which precipitates at a pH of 7.0. Further precipitations with sodium chloride do not effect a greater purification. This moist salt precipitate is dissolved in 95 per cent alcohol, using about 500 cc. for each 96 quarts of original alcoholic extract. The alcoholic extract is filtered or centrifuged to remove some dark insoluble matter which is largely salt. By adding the clear solution to 2 volumes of ether, a precipitate containing all the active material is thrown down. After 24 hours, the supernatant alcohol-ether mixture is drawn off and the ether is removed from the precipitate on a vacuum line.

This precipitate is dissolved in sufficient water, about 1 liter for each 50 quarts of original alcoholic extract. It usually holds enough acid to insure solution. Sodium hydroxide is added to the clear solution until the isoelectric point is reached, pH 4.4 to 5.0. After standing overnight, the liquid is centrifuged and the precipitate is redissolved in approximately $\frac{1}{2}$ the volume of water and the necessary amount of hydrochloric acid. After

the third isoelectric precipitation, the precipitate is dissolved in double distilled, sterile water and made acid with hydrochloric acid to a pH of 3.8 to 4.0.

The potency is then tested on rabbits, after which enough sterile water is added to obtain the desired strength. Phenol is added to a concentration of 0.2 per cent and enough sodium chloride to make the solution isotonic. The liquid is drawn through a Mandler filter and bottled. Bacteriological tests are made to insure sterility. Such preparations appear to keep indefinitely at room temperature without loss of potency.

The yields of crude insulin by this method vary from 1800 to 2500 clinical units per kilo of pancreas.[1] After the third isoelectric precipitation from water, from 1000 to 1400 units with a nitrogen content of about 0.006 mg. per unit are obtained from 1 kilo of material. Small amounts of the insulin remaining in the filtrates from the isoelectric precipitates may be recovered by salting, taking up in water, and precipitating at the isoelectric points. The greater part of this insulin is, however, very difficult to separate in any degree of purity. A rather satisfactory way of doing so is described in a later section under phenol treatment. The method of preparation outlined is a combination of those described by Scott and Best (1) and by Somogyi, Doisy, and Shaffer (2). Sodium chloride instead of ammonium sulfate is used as the precipitant. The use of sodium chloride seems preferable, not only because less salt is required, but because it is convenient to use when one desires to follow the nitrogen distribution of the various fractions. Also there is no danger of introducing sulfates when a study of the sulfur content of insulin is being made.

Purification of Insulin.

(*a*) *Fractional Precipitation with Alcohol.*—A less pure insulin is obtained when but one salt precipitation is made in the method

[1] The unit referred to in this paper is the clinical unit. Lilly's "iletin" U 80,80390–758690 was used as the standard. Our preparations were assayed by the method developed in this laboratory. Sahyun, M., and Blatherwick, N. R., *Am. J. Physiol.*, 1926, lxxvi, 677.

described above. This crude insulin, when dissolved in alcohol, precipitated with ether, and precipitated once from water at the isoelectric point, contains at least two substances. These can be rather completely separated by utilizing their different solu-

TABLE I.

Fractional Precipitation of Insulin with Alcohol.

Experiment No.	Material.	Units.	Nitrogen per unit.
			mg.
I	Before alcohol separation.	28,600	
	Insoluble, 86 per cent alcohol.	14,430	
	Soluble, 86 " " "	16,600	0.0045
II	Insoluble, 86 per cent alcohol.	16,500	
	Soluble, 86 " " "	6,300	
III	Insoluble, 77 per cent alcohol.	7,500	
	Soluble, 77 " " "	16,600	
IV	Insoluble, 77 per cent alcohol.	15,000	0.018
	" 87 " " "	35,000	0.0052
	" 92 " " "	35,000	0.0055
V	Insoluble, 75 per cent alcohol.	10,000	0.0496
	Soluble, 75 " " "	166,000	0.0051
VI	Insoluble, 75 per cent alcohol, pH 6.	10,000	0.059
	" 75 " " " " " 4.6.	9,000	0.050
	Soluble, 75 " " "	83,000	0.0058
	Isoelectric precipitate of above.	83,000	0.0040
VII	Solution of ether precipitate.	355,000	0.014
	Isoelectric precipitate of above.	286,000	0.011
	Insoluble, 75 per cent alcohol.	15,500	0.019
	Soluble, 75 " " "	200,000	0.0048
	Isoelectric precipitate of above.	169,000	0.0038

bilities in alcohol. If one dissolves the above material in a minimum amount of water and sodium hydroxide, adds alcohol to a concentration of about 75 per cent (hydrometer), and adds hydrochloric acid to a pH of about 6.6, a dirty, meaty looking precipitate is formed. This material is found to be relatively

impure and carries most of the brown-red color of the original solution. Upon the addition of absolute alcohol to the filtrate from this precipitate, in amount to make a concentration of 92 per cent alcohol, and upon adjusting to a slightly more acid reaction, a flocculent, white precipitate forms. This material is much purer than the other fraction and carries most of the activity. It has a pale, straw color in dilute acid solution at a concentration of 100 units per cc. Great care must be observed in adjusting the reaction and in employing the correct concentrations of alcohol. The purpose of the separation at 75 per cent concentration is to avoid the isoelectric point of the purer material. The impurities may also be eliminated by adjusting to the acid side of the isoelectric point. If the concentration of alcohol is over 75 per cent, there is danger of losing some of the purer product in the precipitate which forms. The effect of using higher concentrations of alcohol is shown in Experiments I and II of Table I. Typical results of the fractionation are shown in the table. It is the best method we have found for purifying insulin. These observations confirm and extend the findings of Somogyi, Doisy, and Shaffer (2).

(*b*) *Dialysis.*—The material used in these experiments was extracted according to the original Collip method. After concentration and removal of fat with ether, the aqueous solution was saturated with sodium chloride. This precipitate was dissolved in alcohol and again precipitated by adding to ether. The aqueous solution of the ether precipitate was used for the dialysis experiments. Rather thin collodion membranes were used. Before dialysis the solutions were adjusted to barely the alkaline side of the isoelectric point. Dialysis was continued for about 5 hours, with three changes of distilled water. The first experiment showed that 4 per cent of the active material dialyzed. 63 per cent of the potency was found in the isoelectric precipitate of the residue and 33 per cent remained in the filtrate. The biuret reaction of the dialysate, after concentration, was a distinct pink in contrast to the violet color of the isoelectric material.

The second experiment made use of similar material. During dialysis a precipitate formed within the sac. This precipitate was removed and kept separate from the isoelectric material

obtained from the residual fluid. The first material proved to be less pure than the other and was more difficult to purify by

TABLE II.

Isoelectric Precipitation of Insulin After Dialysis.

Experiment No.	Material.	Units.	Nitrogen per unit.
			mg.
I	Residual material after isoelectric precipitation.	21,000	0.0060
	Isoelectric precipitate of above.	21,000	0.0043
	" " " "	21,000	0.0035
	" " " "	21,000	0.0031
II	Residual isoelectric material after removal of precipitate which formed during dialysis.	45,000	0.0065
	Isoelectric precipitate of above.	36,000	0.0050
	" " " "	35,000	0.0045
	" " " "	35,000	0.0036
	Material which precipitated during dialysis.	16,500	0.0140
	Isoelectric precipitate of above.	15,000	0.0103
	" " " "	15,000	0.0061
	" " " "	15,000	0.0052
	" " " "	12,500	0.0040
III	Residual isoelectric material after removal of precipitate which formed during dialysis.	12,500	0.0084
	Isoelectric precipitate of above.	12,500	0.0046
	" " " "	12,500	0.0042
	Material which precipitated during dialysis.	25,000	0.010
	Isoelectric precipitate of above.	22,000	0.0066
	" " " "	22,000	0.0053
	" " " "	22,000	0.0049
	" " " "	20,000	0.0042
	" " " "	20,000	0.0036

the isoelectric method. The isoelectric point of these preparations when purified was about pH 6.0 (colorimetric). The solutions

were colorless in concentrations of approximately 70 units per cc. The experimental results are shown in Table II.

Dialysis enables a separation of two fractions to be made, one of which is purer and is easier to purify further. Probably the chief advantage gained is a sharper precipitation at the isoelectric point, due to the low concentration of inorganic salts. Loss of potency is therefore not so great.

(*c*) *Heating.*—Insulin is precipitable by heating aqueous solutions under certain limited conditions. The pH must be about 4.0, a slight deviation causing either partial or no precipitation; the concentration must be more than 50 units per cc., and the sodium chloride content must be about 1 per cent.

The solution is heated in an air or water bath at a temperature of 80°C. for a period of from 60 to 90 minutes. A precipitate usually begins to form after 15 to 20 minutes heating. It has an entirely different appearance than the ordinary isoelectric precipitate. It forms in a more or less finely divided state making separation by the centrifuge very difficult. Oftentimes it tends to settle as a gel. The precipitate formed under these conditions is acid-insoluble but becomes soluble in acid after having been dissolved in dilute alkali. The material obtained is probably a denatured product which then coagulates under the particular experimental conditions. Sometimes a very great purification is effected, in other cases little, if any, change is brought about. The danger of immediate loss in potency due to heating for the period specified is practically negligible. However, aqueous solutions of these heat precipitates appear to be much less stable under ordinary conditions of light and temperature than the material which has not been so treated. An example will suffice to show the results of this treatment. 2100 units of insulin in a volume of 20 cc. were treated as above. All of the active substance was recovered in the heat precipitate. The nitrogen content was 0.0085 mg. per unit before, and 0.0035 mg. after heating.

(*d*) *Hydrochloride Purification.*—There are several references in the literature to the precipitation of insulin by high concentrations of acid. Maloney and Findlay (3) found that insulin was precipitated by 5 N acid with loss of potency. We have been

able to purify insulin in several instances by using the minimum amount of hydrochloric acid required for precipitation. The greater proportion of the potent material is precipitated with some protein. About 4 per cent of hydrochloric acid is required to bring about complete precipitation. The distribution of the nitrogen is illustrated in Table III.

Experiments I, II, and III were performed in test-tubes. Concentrated hydrochloric acid was added, drop by drop, until no more precipitate formed. The precipitate was separated immediately by the centrifuge. In Experiments I and II there was no loss of potency. In Experiment II practically all of the potency was found in the precipitate with an elimina-

TABLE III.

Precipitation of Insulin with Hydrochloric Acid.

Experiment No.	HCl	Original.		HCl precipitate.		HCl filtrate.	
		Units.	N per unit.	Units.	N per unit.	Units.	N per unit.
	per cent		*mg.*		*mg.*		*mg.*
I		1,000	0.0055	850	0.0044	140	0.011
II		1,000	0.0063	950	0.0042	50	0.048
III		1,100	0.028	550	0.043	80	0.12
IV	4.0	58,000	0.0064	55,600	0.0056	2,000	0.027
V	3.2	55,600	0.0056	40,000	0.0071	1,500	0.022
VI	2.1	40,000		22,000	0.012		
	4.2			5,500	0.010		
VII	2.7	16,500	0.037	3,500	0.054		
	5.4			1,500	0.100		

tion of one-third of the original nitrogen. Experiments IV, V, VI, and VII were performed with larger amounts of insulin, so that the exact acidity might be determined. In these experiments the solution was kept in an ice bath while the precipitation was made. It will be noted that in Experiments III and VII, where the original insulin was quite impure, a large loss of potency resulted. This is contrary to the usual behavior of insulin and may have been accidental. Experiments V and VI were a continuation of Experiment IV. In Experiment IV, a purification with no loss of potency resulted. When the hydrochloric acid precipitation was repeated, 30 per cent of the potency was lost. We do not believe that the degree of purification sometimes resulting from a hydrochloric acid precipitation overbalances the attendant risk of destroying the active substance.

(*e*) *Precipitation with Pyridine.*—The precipitation of insulin by pyridine resembles any other precipitation at the isoelectric

point. Pyridine is convenient to use because of its weak alkalinity. A considerable excess of the reagent must be added before solution of the precipitate occurs. In one instance a 3 per cent solution of pyridine in water was required to dissolve a sample of insulin. This insulin was soluble in 82 per cent pyridine, but it was insoluble in greater concentration. We have had similar results with other organic bases, such as aniline and benzidine. In the case of aniline, which is a much stronger base than pyridine, a slight excess will redissolve the precipitate. The pH of the filtrates from the pyridine precipitates is the same as the isoelectric point of the insulin used. It has been repeatedly demonstrated in this laboratory that after several precipitations at the isoelectric point, no further purification can be obtained by this procedure. The same is true with the pyridine precipitation. 25,000 units of insulin, with a nitrogen content of 0.038 mg. per unit, were subjected to five pyridine precipitations and to two isoelectric precipitations using sodium hydroxide to remove the pyridine nitrogen. 16,000 units with a nitrogen content of 0.032 mg. per unit were obtained. In another instance the nitrogen was reduced from 0.016 to 0.014 mg. per unit by two pyridine precipitations. The degree of purification is the same in both cases.

(*f*) *Phenol Treatment.*—Abel and Geiling (4) have made use of the fractional solubility of insulin in phenol to effect purification. They found that the phenol-insoluble residue contained very little if any potency. In using their method upon impure insulin residues, an additional separation of impurities may be obtained by somewhat altering the conditions of precipitating the insulin when the phenol is added to the water. When the phenolic solution is added to the minimum amount of water (15 parts water to 1 part phenol), a colloidal solution and a precipitate are formed. The precipitate is centrifuged off. The colloid contains more than half the potency in a purified form. The colloidal solution can only be separated in part by the Sharples supercentrifuge. This material is recovered from the phenolic liquid by saturating with salt. The following example illustrates the treatment. 71,000 units of insulin, with a nitrogen content of 0.11 mg. per unit, were dissolved in 59 gm. of phenol. In

this instance the substance was completely phenol-soluble. The colloidal solution, obtained by adding to water and centrifuging off the sticky precipitate, was run through the supercentrifuge. 25,000 units, with a nitrogen content of 0.050 mg. per unit, were found in colloidal solution. 22,000 units, with a nitrogen content of 0.028 mg. per unit, remained in the precipitate retained in the supercentrifuge. The colloidal solution of insulin, mentioned above, is not always found.

The phenol treatment has been used in recovering the insulin from the impure residues of the manufacturing process. The insulin remaining in these solutions is salted out. Attempts at recovering insulin from this dried precipitate, which contains from $\frac{1}{2}$ to 1 unit per mg., were unsuccessful until the phenol technique was applied. A phenol separation, followed by a fractional alcohol precipitation, gives a good insulin, but the yields are low. 12,000 units, with a nitrogen content of 0.0063 mg. per unit, were obtained from 71,000 units having a nitrogen content of 0.11 mg. per unit. The following procedure was used.

The dried salt precipitate is powdered. 1 part by weight of powder and 2 parts of absolute phenol are heated together for 1 hour in a water bath at a temperature not to exceed 60°C. Most of the powder dissolves. The solution is centrifuged and the clear supernatant liquid is added to exactly 30 parts of water. In doing this it is well to have the water under agitation and then add the phenol drop by drop. The precipitate which forms is separated by centrifuging. It is discarded. The supernatant liquid is salted, whereupon a tarry residue separates. This residue is subjected to two isoelectric purifications. Precipitates may be found at more than one pH. These precipitates are combined. The final purification is the fractional alcohol precipitation described above. The precipitate which forms at a concentration of 75 per cent alcohol is very impure and is discarded. The soluble fraction contains the purified insulin which may be separated completely by the addition of more absolute alcohol and ether.

Action Of Various Chemical Reagents upon Insulin.

(*a*) *Hydrogen Sulfide.*—Murlin (5) was able to inactivate insulin by exposing it to hydrogen sulfide and to reactivate it by subsequent exposure to air or oxygen. This we were unable to do. A very impure insulin analyzing 1 unit per mg. was used in the first experiment and no inactivation was found. Exposures of a purer insulin, assaying 0.0055 mg. N per unit, resulted in the

complete and irreversible inactivation of the insulin. These preliminary experiments indicated the presence of a protective

TABLE IV.

Effect of Hydrogen Sulfide in Solution on Insulin.

Nitrogen per unit.	Medium.	Concentration.	pH	Time.	Original potency.	Recovered potency.
mg.				*hrs.*	*units*	*units*
0.0055	0.001 N H_2S.	100 units in 50 cc.	6.2	24	100	0
0.0055	0.001 " "	100 " " 50 "	6.0	24	100	0
0.0055	0.010 " Congo red.	100 " " 50 "		24	100	100
0.0055	0.010 " H_2S. 0.010 " Congo red.	100 " " 50 "	*	24	100	25
0.0055	0.005 " H_2S. 0.010 " Congo red.	100 " " 50 "		84	100	55
0.0055	0.001 " H_2S.	200 " " 50 "		48	200	100
0.0055	0 005 " " 0.5 per cent casein.	200 " " 50 "		48	200	165
0.0055	0.005 N H_2S.	50 " " 50 "		48	50	12
0.016	0.001 " "	100 " " 50 "	7.8	24	100	100
0.016	0.001 " "	100 " " 50 "	5.6	72	100	75
0.070	0.001 " "	200 " " 50 "	8.0 to 4.5	24	200	180
0.070	0.010 " "	200 " " 50 "	4.0	24	200	100

* The pH of the Congo red solutions was adjusted so that the dye was at the transition between the blue and red forms.

substance in the impure insulin and they have led to a study of the effect of the purity upon the stability.

Action of Sulfide in Aqueous Solution.—We used insulins of

varying degrees of purity, analyzing respectively 0.0055, 0.016, and 0.070 mg. of nitrogen per unit. In 0.001 N sulfide solution adjusted to a slightly acid pH, the insulin, analyzing 0.0055 mg. of N per unit, was completely inactivated in 24 hours. Under the same conditions, the 0.016 mg. of N insulin retained three-fourths of its activity after 72 hours, and the 0.070 mg. of N insulin, 90 per cent of its activity after 24 hours. In another experiment similar results were obtained with a 0.01 N solution but the odor of sulfide was strong after 72 hours. This invalidated the possibility that the impurities of the insulin functioned by destroying the hydrogen sulfide. The calculated amount of hydrogen sulfide in solution required to inactivate completely a

TABLE V.

Effect of Hydrogen Sulfide Gas on Dry Insulin.

Experiment No.	Nitrogen per unit.	Time.	Treatment.	Potency in units per mg.	
				Before.	After.
	mg.	*hrs.*			
I	0.16	24	Dry H_2S atmosphere.	0.9	0.9
II	0.0055	48	" " "	22.0	15.0
III			Material treated with H_2S in Experiment II. Exposed to air current for 24 hours.	15.0	14.7
IV	0.0033	24	Dry H_2S atmosphere.	39.0	16.0
V			Material treated with H_2S in Experiment IV. Exposed to air current.	16.0	12.0

given amount of highly purified insulin is 0.7 mg. of sulfide for 1.5 mg. of insulin. 50 cc. of 0.001 N sulfide solution will completely inactivate 100 units of this insulin. If 200 units are tested, only half will be inactivated.

Action of Hydrogen Sulfide Gas on Dry Insulin.—Three samples of insulin which analyzed 0.16, 0.0066, and 0.0033 mg. of nitrogen per unit were used. Before being brought into contact with the powdered insulin, the hydrogen sulfide was dried by passing it through calcium chloride. A steady stream of the gas was conducted over the insulin powder for an hour. The container was then stoppered so that the insulin remained in an atmosphere of hydrogen sulfide. Under this treatment the very impure

insulin lost none, the fairly pure insulin lost one-fourth, and the highly purified insulin over one-half of its activity. Exposure to air for days did not reactivate the powders which had been partly inactivated by the hydrogen sulfide. An interesting observation was made. After the highly purified insulin had been subjected to the stream of hydrogen sulfide, the stopper of the container was accidentally opened, diluting the sulfide atmosphere with air. No inactivation resulted. On repeating the experiment without the introduction of air, over half the potency was destroyed. This indicates that the inactivation is preceded by an adsorption and that it is necessary not only to remove the adsorbed air from the adsorbing centers, but to keep it away while the adsorbed hydrogen sulfide is functioning in its destruction of the active group.

Since the experiments both in gas-solid phase and solution demonstrated the protective action of the impurities in the insulin, an attempt was made to find an artificial protective agent, which, when added to a very pure insulin, would inhibit the destruction of the insulin by hydrogen sulfide. The substantive dye, Congo red, was found to have this property. Congo red has several properties in common with the proteins; particularly its high adsorbing powers, the bulk of its molecule, its amphoteric nature, containing the basic amino and acidic phenolic and sulfonic acid groups, and its colloidal state of aggregation in alkaline solution. The highly purified insulin is completely inactivated by 0.001 N sulfide solution. In the presence of 0.01 N Congo red solution less than half the activity was destroyed by 0.005 N sulfide solution, 5 times the amount which would have normally destroyed it. Allen and Murlin (6) report that the biuret-free preparations they obtain from pancreas perfusates are extremely unstable, the activity being lost spontaneously within a week and very often within a few days. The chemical study of these highly purified insulins is rendered even more difficult than was first anticipated because of the great instability of the purified insulin. The results of our experiments with Congo red suggest the possibility of stabilizing such insulins by the addition of colloids of known constitution, a procedure which may be likened to the protection of amino groups by acetylation.

The addition of a foreign protein, such as casein, was also found to exert a protecting influence.

From the foregoing experiments, we may conclude that the inactivation of insulin by hydrogen sulfide is dependent upon the impurities associated with the insulin, very pure insulin being entirely inactivated and crude insulin being unaffected. With the insulins used in this laboratory, the inactivation was irreversible. From this instance as well as from others discussed in this paper, we do not intend to infer that insulins obtained by other workers will not react differently. The addition of a protective colloid to a purified insulin increased its stability to the destructive action of hydrogen sulfide.

Experimental.—The insulin, analyzing 0.0055 mg. of N per unit, was prepared by the routine procedure of this laboratory. That analyzing 0.016 mg. of N per unit was made, using Abel's (4) phenol, pyridine purification upon impure insulin residues. The very impure insulin was the "insulase" of Armour and Company, which is no longer on the market. The dry insulin, with a potency of 39 units to the mg., was prepared by an isoelectric precipitation of the insulin analyzing 0.0055 mg. of N per unit.

(*b*) *Cyanide.*—Insulin gives a negative test with nitroprusside solution, showing the absence of mercaptan groups. However, when it is treated with cyanide and then subjected to nitroprusside, a very distinct, positive test results. This confirms the observation of Scott (7) and others, that insulin contains cystine. The function of the cyanide (Walker (8)) is to reduce the disulfide cystine sulfur to the mercaptide cysteine linkage.[2] 4 mg. of insulin, containing 0.0053 mg. of nitrogen per unit, were used for a test.

Since cyanide attacks the insulin complex and reduces the cystine, it was interesting to determine what effect such treat-

[2] It has been found advantageous to modify Walker's procedure. He adds the nitroprusside first, then the cyanide. We found the sensitivity of the test was increased by reversing the order. The excess of cyanide apparently reacts with the nitroprusside, as is shown by the disappearance of the normal yellow color of the dilute nitroprusside, necessitating the addition of an excess of the latter reagent. When the cyanide is added after the nitroprusside, as Walker directs, an inappropriate balance between nitroprusside and cyanide may result, and a negative test is obtained in the presence of cystine.

ment would have upon the potency. It was found that insulin which had been exposed to the action of cyanide for 18 hours was completely inactivated. When the insulin was salted out and separated from the cyanide solution by centrifugation, it gave a positive nitroprusside test without the addition of cyanide to the solution. The experiment was repeated, exposing the insulin to the action of cyanide for only 15 minutes, when it was separated from the cyanide solution by an isoelectric precipitation. 30 per cent of the original potency was recovered. By reducing the concentration of the cyanide, 60 per cent of the potency was recovered. The insulin, which was completely inactivated by long contact with cyanide, was partly acid-insoluble. The results of these experiments indicate that the cyanide reacts with the insulin complex in more than one way; the reduction of the cystine to cysteine takes place instantly, the destruction of the groups connected with the potency more slowly, and the denaturing of the protein to an acid-insoluble form very gradually.

Experimental.—0.1 gm. of sodium cyanide in 1 cc. of water was added to 1000 units of insulin, analyzing 0.0055 mg. of N per unit, in 5 cc. of water. The pH of the solution was 7.0. After 15 minutes the solution was made faintly acid, saturated with sodium chloride, and centrifuged. The precipitate was dissolved in 10 cc. of water. 1 cc. gave a positive nitroprusside test without the addition of cyanide. 350 units were recovered. The experiment was repeated, subjecting the insulin to the influence of the cyanide for 18 hours. No potency was left. In a third experiment 0.1 gm. of sodium cyanide was added to 90 units of insulin in 50 cc. of water. After 15 minutes the solution was made acid and saturated with salt. 50 units of insulin were recovered. 50 units will give the nitroprusside test, but the test is not sensitive to smaller amounts. The possibility that the alkalinity of the sodium cyanide caused the destruction of the potency must be considered. A sample of this insulin dissolved in 0.1 N sodium carbonate lost three-fourths of its potency in 90 hours. This was a greater degree of alkalinity than obtained in the experiments with cyanide.

(*c*) *Nitrite.*—Scott (7) has studied the effect of nitrous acid upon insulin. Under the conditions of his experiments in which a very large excess of nitrous acid was used (1 gm. of nitrite for 20 mg. of insulin), inactivation resulted. He used acetic acid as his solution medium. By using very dilute aqueous nitrous

acid solutions, we have been able to affect the insulin complex without destroying the potency. Not only the amount but the concentration of the nitrous acid is an important factor in regulating its destructive effect upon the potency. Insulin is stable in a 0.002 N nitrous acid solution. In a 0.05 N solution, from 15 to 30 per cent of the potency is destroyed and the product becomes nearly acid-insoluble. The biuret test remains positive. Nitrous acid has a marked effect upon the labile sulfur of the insulin. This will be considered in that section of the paper devoted to labile sulfur. In concentrated solution nitrous acid is unstable, decomposing to various oxides of nitrogen. The inactivation of insulin by concentrated nitrous acid solutions is no doubt due to the oxidizing effect of these decomposition products. From a structural point of view, it is important to know that the active insulin group does not react with nitrous acid itself.

Experimental.—100 units of insulin, having a nitrogen content of 0.0033 mg. per unit, 1 drop of 10 per cent hydrochloric acid, and 3 mg. of sodium nitrite were made up to 25 cc. and kept below 5°C. for 12 hours. The pH of the mixture was 3.4. The solution was used for rabbit tests without further adjustment. None of the potency was destroyed.

1000 units of insulin, having a nitrogen content of 0.0055 mg. per unit, 2 drops of 10 per cent hydrochloric acid, and 30 mg. of sodium nitrite were permitted to react for 4 hours at a dilution of 10 cc. A yellow precipitate formed. The insulin was salted out, taken up in 25 cc. of acidulated water, to which a drop of concentrated ammonia was added to destroy any occluded nitrous acid. 30 per cent of the potency was destroyed. This preparation was used for labile sulfur analyses. Hence the precaution to remove all traces of nitrous acid.

The conditions of the above experiment were repeated, but the excess nitrous acid was removed after about an hour. Only 15 per cent of the potency was destroyed. 80 units of this treated material gave a strong biuret test. The solution gave a negative test with starch-iodide solution after separation from the excess nitrite. The major portion of the insulin was acid-insoluble.

(*d*) *Diazo Reaction.*—Shonle and Waldo (9) found that a highly purified insulin, prepared by dialysis and containing 0.002 mg. of nitrogen per unit, gave a positive Pauly (Ehrlich) diazo test with an intensity per unit proportionate to the original preparation. Scott (7) reports a positive Pauly test on insulin analyzing

0.006 mg. of nitrogen per unit. This insulin analyzed 10 per cent tyrosine by the Folin-Looney method. Since the test is characteristic for tyrosine and histidine, and for proteins containing these amino acids, it was interesting to determine whether insulin, which had been coupled with a diazo component—not necessarily the diazobenzenesulfonic acid of Ehrlich—would retain its potency. If the physiological activity of insulin is characteristic of some peculiar chemical grouping (structure), then the addition of a group such as the azo component to another part of the molecule should not affect this property. If the physiological activity of the insulin complex is not solely dependent upon some specific atomic grouping, but requires specific conditions for adsorption, or osmosis, or some other more physical property, then the addition of another component to the active molecule might so change the physical properties of the entity as to render it physiologically inactive. Inactivation of the insulin molecule would also result if the active chemical group were destroyed by the diazo compound, either by combining with it, or through an oxidation or reduction reaction.

Since the Pauly diazo test, as ordinarily carried out, would be subject in this instance to many side reactions, the conditions of experimentation were modified to eliminate as many of these influences as possible. Insulin is very sensitive to alkali. In the test as given by Mathews the mixture should be made distinctly alkaline with ammonium hydroxide or sodium carbonate. We found that the coupling takes place in very faintly alkaline solution. It was therefore unnecessary to make the insulin strongly alkaline and thus risk the destruction of most of the potency. A large excess of nitrous acid is used in diazotizing the sulfanilic acid in the Pauly test. It has been shown that concentrated nitrous acid greatly decreases the activity of insulin. In these experiments the minimum amount of nitrite (105 per cent of that required by theory) was therefore used. The coupling was carried out in ice water to eliminate the side reactions caused by heat.

Our experiments with diazo compounds have led to the following conclusions. At an acid pH insulin is stable to dilute solutions of diazonium salts. More concentrated solutions destroy

some potency. The same results were obtained with nitrous acid. In faintly alkaline solution coupling takes place, and the potency is destroyed. The amount of diazo required to destroy the insulin is considerably less than the theoretical amount required to couple with the histidine and tyrosine. Thus, 2 cc. of 0.002 N diazo solution destroyed 85 per cent of an original 200 units of insulin. On the basis of Scott's figures for the histidine and tyrosine content of insulin, 6 cc. would have been required for a complete coupling. In this inactivation, a considerable portion of the diazo was consumed in coupling, as shown by the development of a decided coloration. Although not a limiting value, because of the fact that the inactivation and coupling take place simultaneously, the value nevertheless indicates that a very small per cent of the insulin complex constitutes the active insulin group. The alkaline azo coupling completely destroys the labile sulfur. This will be considered in the section devoted to that subject.

In making a colorimetric comparison between diazotized insulin and casein, values for tyrosine and histidine which approximate those of other workers were obtained. Insulin developed a color of 1.6 times the intensity that an equal weight of casein gave. The shade of the color was exactly the same, indicating that the distribution between histidine and tyrosine in insulin and casein is approximately the same. Scott reports 10 per cent tyrosine and 5 per cent histidine. We obtained 14 per cent tyrosine and 8.5 per cent histidine colorimetrically, and 17 per cent tyrosine by the Folin-Looney method. The insulin azo compound is acid-insoluble and alkali-soluble when the azo component contains a sulfonic acid group, like diazobenzenesulfonic acid. The azo compound with tetrazodiphenyl (benzidine) is neither acid- nor alkali-soluble.

Experimental.—A few of the more important experiments are recorded.

5 cc. of a diazobenzene solution, made by adding 0.1 cc. of N nitrite solution to 0.5 gm. of sulfanilic acid and 1 cc. of hydrochloric acid in 100 cc. of ice water, were added to 2000 units of insulin, having a nitrogen content of 0.0055 mg. per unit. After 10 minutes the solution was saturated with salt and the insulin separated from the diazo liquor by the centrifuge. It was made to 25 cc. volume with dilute alkali and then reacidified. 75 per cent of the potency was recovered. After making alkaline, it became partly

acid-insoluble and developed a slight yellow color. This was probably due to a small amount of occluded diazo compound. The preparation was used for the labile sulfur analyses.

1000 units of the same insulin used in the above experiment were treated with an excess of diazobenzenesulfonic acid in sodium bicarbonate solution for 1 hour in an ice bath. The solution was then made acid and saturated with salt, so that the insulin azo compound might be centrifuged. Although acid-insoluble, this compound is so finely divided when first formed that it will not separate completely without the addition of salt. The potency was completely destroyed. Doses representing 20 original units failed to lower the blood sugar of rabbits.

A 0.002 N diazobenzenesulfonic acid solution was made by adding 120 per cent of the theory of required nitrite to the theoretical amount of sulfanilic acid and dilute hydrochloric acid in a volumetric flask cooled with ice. The solution was placed in the ice box for 4 hours to insure a complete diazotization. 1 and 2 cc. respectively of this diazo solution were added to 200 units of insulin in 5 cc. of phosphate buffer having a pH of 6.8. The solutions were made alkaline for 30 seconds by adding a drop of sodium hydroxide. They were then reacidified. The final dilution was 25 cc. The sample treated with 2 cc. of diazo solution lost over 85 per cent of its potency. 1 cc. of diazo solution destroyed about 75 per cent of the potency. Both samples were highly colored in alkaline solution.

Solutions of 0.0038 gm. of insulin, representing 0.0055 mg. of N per unit, and 0.0060 gm. of casein were placed in 100 cc. volumetric flasks with 1 gm. each of sodium carbonate. The volume was made up to about 75 cc. and 0.25 gm. of diazotized sulfanilic acid was added to each. They were diluted to the mark and placed on ice overnight. The insulin had just twice the color intensity of the casein solution.

(*e*) *Iodine.*—Brand and Sandberg (10) have suggested a possible iodometric titration of insulin. Their results seem to indicate that if the insulin preparations are of sufficient purity, the number of units can be established iodometrically by using a neutral, buffered, iodine-potassium iodide solution. They used the standard insulin preparations of Lilly, Toronto, and Squibb and found a surprisingly close agreement between cc. of iodine absorbed and number of units represented. We have performed their titration and a more rapid modification on several of our insulin materials prepared or purified in various ways. These results are summarized in Table VI. While there is a very close agreement for seven of the nine samples represented, two of them, Insulin IV and resin from Insulin X, although representing the same degree of purity, indicate a much lower capacity

for iodine absorption. When Insulin IV was one-half destroyed by exposure to ultra-violet light, it still gave the same iodine value. These results indicated that the iodine reaction in part involved groups of the insulin complex other than the active insulin group. To establish this point, the minimum amount

TABLE VI.

Amounts of 0.005 N Iodine Required to Saturate 1 Unit of Insulin.

Insulin.	Nitrogen per unit.	pH 4.5 2 hrs. at 37°C.	pH 4.5 16 hrs. at 37°C.	pH 10.0 5 min. in ccld.	pH 6.8 16 hrs. at 37°C.
	mg.	*cc.*	*cc.*	*cc.*	*cc.*
II, dialysis	0.0069	0.011	0.021	0.077	0.054
III, alcohol purification	0.0043	0.0085	0.019	0.073	0.059
IV " "	0.0055	0.013	0.016	0.062	0.046
IV, half destroyed by ultra-violet light.				0.062	
HCl purified	0.010			0.078	0.060
75 per cent alcohol-insoluble	0.012			0.073	
Iletin 80390–758690	0.0063			0.058	0.053
X, resin	0.0043			0.033	0.031
X, flocculent	0.0056			0.070	0.057

TABLE VII.

Inactivation of Insulin with Iodine.

Experiment No.	Reaction.	0.005 N iodine.	Units recovered.
	pH	*cc.*	*per cent*
I	8.0	9.3	0
II	8.0	3.7	0
III	8.0	1.9	10
IV	6.8	3.7	0
V	6.8	0.93	25

200 units of insulin were used in each experiment. 1 unit weighed 0.038 mg. The following amounts of iodine were required to saturate 1 unit of insulin: at a pH of 6.8, 0.0295 mg; at a pH of 8.0, 0.0393 mg. At a pH of 8.0, 0.005 mg. were required to inactivate 1 unit of insulin.

of iodine to inactivate a definite number of units was determined. Only one-seventh the iodine that was absorbed was required to inactivate. The exact figures are given in Table VII. Brand and Sandberg found that the iodine destroyed the lead-blackening sulfur of insulin. We have found that about 50 per cent of the

labile sulfur in insulin has no connection with the potency. Iodine reacts with cystine and such phenolic bodies as tyrosine. Different insulin preparations contain varying amounts of these amino acids. Considering these facts, one must dismiss the feasibility of a determination of potency by an iodometric titration.

Experimental.—The reactions were performed in ground glass, stoppered bottles to prevent loss of iodine by vaporization. 0.01 N iodine and 0.005 N thiosulfate solutions were used. From 20 to 40 units of insulin, 5 cc. of phosphate buffer, 5 cc. of iodine solution, and 25 cc. of water were permitted to react under the conditions given in Table VII. A blank was always run simultaneously and the value of the iodine was taken from this figure. At a neutral or acid pH, the absorption varies with the time. At a pH of 6.8, 200 units of insulin absorbed 1.1 cc. of iodine solution in 5 minutes, 1.6 cc. in 40 minutes, and 2.0 cc. in 65 minutes. We found it very convenient to make a rapid titration in the following way. All the materials are added together in a pH 6.8 phosphate buffer. Sodium hydroxide is then added drop by drop until the iodine color disappears. After 5 minutes, acetic acid is added and the excess iodine determined. It will be noticed that the values obtained in this way are just as consistent as those obtained by the neutral incubation method.

(*f*) *Benzoylation.*—Scott (7) and Dingemanse (11) both report experiments on the benzoylation of insulin. Using the Schotten-Baumann technique they obtain an ether-, acid-, and alkali-insoluble product with almost complete loss of potency. In a personal communication[3] Scott writes that in one instance he was able to reactivate the insulin through an acid hydrolysis. By using large amounts of benzoyl chloride, we have always obtained complete inactivation. When the amount of the benzoyl chloride was reduced, part of the potency was recovered

[3] "I have just looked up my laboratory notes on the benzoylation experiments. In these experiments I find that I had a precipitate settle out which was ether-, acid- and alkali-insoluble. In my published article I should have made this clear as the inference is that everything is acid-soluble. I did a great deal of work in trying to activate the different fractions after treatment with benzoyl chloride. In only one experiment out of a great number was I successful. In this experiment after removing the precipitate caused by the benzoylation I hydrolyzed the supernatant liquid with dilute hydrochloric acid in the usual way. I obtained a very active product which caused convulsions in rabbits. Numerous attempts to repeat this experiment were made, but with no success."

in the form of an acid-insoluble, alkali-soluble product. On standing a few days, the product lost its potency. Benzoyl chloride reacts with hydroxy, amino, and amido groups. It also dehydrates. Scott's reactivation experiment and the one in which we obtained an acid-insoluble, potent material appear promising. By modifying the conditions of experimentation, we hope to obtain larger yields of a more stable product.

Experimental.—1600 units of insulin, with a nitrogen content of 0.004 mg. per unit, were diluted to a volume of 40 cc. 1 gm. of sodium bicarbonate and 2 drops of benzoyl chloride were added and the solution shaken until the odor of benzoyl chloride had disappeared. The insoluble product was filtered off. It gave a faint biuret test and contained no potency. The filtrate was rendered acid and extracted with ether 3 times. The acid-insoluble, alkali-soluble, ether-insoluble residue had a potency of 300 units. On the following day the potency had dropped to 125 units and the 3rd day not more than 50 units were left. This product gave a strong biuret test.

1000 units of insulin were shaken, in a 50 cc. dilution, with 0.5 cc. of benzoyl chloride and 1 gm. of sodium carbonate for ½ hour. The mixture was made acid and extracted with ether to remove benzoic acid. The acid-insoluble residue in the aqueous layer was filtered off. It was alkali-insoluble and contained no potency. Attempts to hydrolyze this compound by heating in 90 per cent phenol at 70°C. were unsuccessful.

Sulfur Content of Insulin, and Other Proteins.

From a study of the labile sulfur content of insulin preparations, Abel is inclined to believe that the "remarkably labile form" of sulfur is an essential constituent of the active hormone. This view is based upon the following evidence. The amount of more (0.1 N sodium carbonate) and less (10 per cent sodium hydroxide) labile sulfur found in insulin preparations is proportional to the unitage. Liberation of the sulfur by heating with phenol or with alkalies is accompanied by inactivation. Abel suggests that the only alternative interpretation of his findings is to assume that the insulin hormone is adsorbed upon a compound containing this labile sulfur. Abel's proposition, "Is insulin an unstable sulfur compound?" must be regarded as a contribution of the greatest importance because it was the first clue to any correlation between potency and chemical activity. It occurred to us that some light might be shed upon the subject by studying the effect of a whole series of chemical reagents upon

the labile sulfur. If the labile sulfur could be destroyed without affecting the potency, then the hormone would not be dependent upon the labile sulfur for its physiological activity. The converse of this proposition was not necessarily true, it being quite possible to destroy other chemical groups necessary for potency without affecting the labile sulfur group. Evidence indicating that the potency was destroyed whenever the sulfur was liberated would lend support to the theory that insulin is a labile sulfur compound. Abel's experiment of heating with phenol belongs in this category.

TABLE VIII.

Relationship Between Purity of Insulin and Labile Sulfur.

Insulin.	Units per mg.	Nitrogen per unit.	0.1 N Na_2CO_3 labile sulfur per unit.	0.1 N Na_2CO_3 labile sulfur.	10 per cent NaOH labile sulfur.
		mg.	*mg.*	*per cent*	*per cent*
Brucine separation	55	0.0026	0.00051	2.8	3.6
I, purified	47	0.0030	0.00034	1.6	2.7
I	37	0.0038	0.00024	0.9	1.3
III	33	0.0043	0.00055	1.8	2.4
X, resin	33	0.0043	0.00055	1.8	2.7
Biuret-free	20	0.005	0.00175	3.5	6.4
IV	26	0.0055	0.00050	1.3	2.4
X, flocculent	26	0.0056	0.00049	1.3	2.3
Biuret-free	10	0.006			1.8
II, isoelectric	25	0.0057	0.00056	1.4	1.9
II, sac	21	0.0069	0.00066	1.4	1.7
Impure	3	0.045	0.02	0.65	1.4

We have been able to show that a large percentage of the sodium hydroxide labile sulfur, the amount representing the difference between the sodium carbonate and sodium hydroxide labile sulfur, is not involved in the potency. This was done by destroying the sulfur with very dilute nitrous acid without affecting the activity. We have never been able to destroy the sodium carbonate sulfur without destroying the potency. By using minimum amounts of iodine, we have destroyed the insulin without changing the sodium carbonate labile sulfur. Since an excess of iodine inactivates this labile sulfur, we have evidence

against the labile sulfur theory. There must be present in insulin a group more sensitive to iodine than the sodium carbonate labile sulfur. In Table VIII a comparison is made between the purity and the sodium carbonate labile sulfur content of twelve different insulin samples. It will be noted that in six of the twelve cases, the sulfur per unit is the same. This agrees with the finding of Abel. In the other five, excepting the very impure insulin, there is either considerably less or more sulfur per unit. The biuret-free insulin is very high in labile sulfur. If the labile sulfur is connected with the potency, limiting values should be obtained as the purity is increased. This is obviously not the case. It happens that under similar processes of purification, the unitage of the product may be proportional to the labile sulfur, or the iodine or peroxide titrations.

While our evidence does not support the labile sulfur theory, it contributes to our knowledge of the chemistry of the labile sulfur in the associated compounds. It has occurred to us that the labile sulfur may serve as a protective agent for the insulin. Both insulin and labile sulfur are very sensitive to chemical reagents. It is possible that, in the metabolism of the insulin, there arise conditions in which the labile sulfur, by reacting with a destructive agent, saves the insulin from inactivation.

More and Less Labile Sulfur in Proteins.—The term labile sulfur, which is found throughout the protein literature, is usually associated with the test by which it is detected; *viz.*, the formation of the black sulfide of lead when the substance is heated with alkali and a soluble lead salt. The labile sulfur may be regarded as that form of sulfur liberated from the protein as a sulfide by heating with alkalies. The detection as the sulfide of lead is a convenient way to inhibit the oxidation by air during the process of liberation. Abel and Geiling have found "that when one is dealing with a substance containing labile sulfur, one may be seriously mistaken in respect to its sulfur content if one makes the qualitative tests for sulfur in one way rather than in another." If the lead salt is added after boiling, all the sulfide may have been destroyed by oxidation and negative results will be obtained. Lead sulfide itself, in a finely divided state, is not immune to oxidation and may be destroyed under certain conditions of experimentation. The only safe way to make the test

is to use an atmosphere of inert gas. In our labile sulfur determinations we have eliminated the use of lead salt. The sulfide is liberated in an atmosphere of inert gas, and swept over into an oxidizing medium where it is determined as the stable sulfate. Piper, Allen, and Murlin (12) found that, "Heated with lead acetate and NaOH there is a slight browning of the fluid which perhaps indicates the presence of a trace of sulfur as an impurity." Yet we find that biuret-free insulin prepared according to Murlin's procedure contains 7 per cent labile sulfur. The discrepancy emphasizes the warning of Abel and Geiling quoted above.

Because we have been led to regard the labile sulfur in insulin as associated with the adsorbing proteins, a number of labile sulfur determinations has been made on several proteins under the identical conditions used for our insulin determinations.[4] There is much labile sulfur data in the literature but no two workers use the same conditions, so that the results are not comparable. The alkalinity used varies anywhere from 0.1 N carbonate to 30 per cent sodium hydroxide, and the time of heating from a few minutes to 9 hours. Johnson (13) gives an excellent summary of the labile sulfur literature in his article entitled "Sulfur linkages in proteins." We find that the labile sulfur in the insulin proteins is no more sensitive to alkalinity than the labile sulfur in keratin and zein. Gelatin and casein contain only a trace of sodium carbonate labile sulfur. The gelatin sulfur is remarkably stable even to 10 per cent sodium hydroxide. We were unable to determine the sodium carbonate labile sulfurs of lactalbumin and cottonseed globulin, because of the partial insolubility of our samples in this reagent. The sodium hydroxide sulfur of these substances is about half the total sulfur. Osborne (14) reports 13.0 per cent of the casein sulfur as loosely bound. This agrees with our sodium carbonate figure. We obtain a much higher percentage of labile sulfur in zein than is reported by Osborne. Our sodium hydroxide figure equals that for total sulfur. As has been stated, a direct comparison of results with other workers is not possible because of the difference in conditions of experimentation.

[4] We are indebted to the Protein Investigation Laboratory of the Bureau of Chemistry for a generous supply of purified proteins.

What do the more and less labile sulfur figures mean? Does the increased alkalinity break down a more stable sulfur or does it merely increase the speed of liberation of the same kind of sulfur linkage? In the case of cystine, we found that under our conditions of experimentation no cystine sulfur is liberated by 0.1 N sodium carbonate and only 2 per cent, or less than 10 per cent of the total sulfur, by sodium hydroxide. Longer heating set free increasing amounts of cystine sulfur. At least 90 per

TABLE IX.

Sulfur Content of Several Proteins and Related Compounds.

Substance.	Sulfur.		
	0.1 N Na_2CO_3 labile.	10 per cent NaOH labile.	Total.
	per cent	*per cent*	*per cent*
Cystine	Trace.	2.0	
Gelatin	"	0.005	0.7*
Casein	0.1	0.2	0.8
Lactalbumin		0.8	1.73*
Cottonseed globulin		0.3	0.62*
Keratin	2.2	2.7	5.0*
" azo	1.4	1.8	
Zein	0.4	0.7	0.6*
Insulin protein 3 units per mg	0.65	1.4	1.8
" purified 50 " " "	2.8	3.6	4.0
" biuret-free 20 units per mg	3.5	6.4	

* Figures taken from literature.

cent of our labile sulfurs are therefore not due to cystine. If the remaining labile sulfur in proteins is of a single type, and the differences obtained with different concentrations of alkali are only a question of degree of reaction, then the ratios of the sulfurs obtained for different concentrations under otherwise identical conditions should be the same. A comparison of the results for keratin, zein, an insulin protein, and a purified insulin shows that this is not the case. By coupling an insulin and keratin with excess of diazo we were able to destroy all the insulin labile sulfur and only a fraction of the keratin labile sulfur. These results indicate that there are at least two other forms of labile sulfur in addition to the cystine form. See Table IX.

Effects of Various Reagents on the Sulfur of Insulin.—The effects of benzoyl chloride, acid and alkaline diazo compounds, phenylhydrazine, ultra-violet light, precipitation by heat, destruction by heat, trypsin, nitrous acid, and iodine upon the potency and sulfur were tried upon the same preparation of insulin. See

TABLE X.

Effect of Various Reagents on the Labile Sulfur of Insulin.

Reagent.	Potency destroyed.	Sulfur.		
		0.1 N Na_2CO_3 labile.	10 per cent NaOH labile.	Total.
	per cent	*per cent*	*per cent*	*per cent*
Insulin IV, 0.0055 mg. N per unit.....	0	1.3	2.4	3.8
" benzoyl.....	100	0.5	0.8	
" azo.....	100	0.2	0.3	
" " acid.....	25	1.1	1.6	
" phenylhydrazine.....	60	1.3	1.6	
" ultra-violet, H, 40 hrs.....	75	1.0	1.6	
" " " 80 "		0.5		
" " N, 17 "	48	0.8	1.2	
" " " 32 "	65	0.9	1.4	
" " " 48 "	75	1.2	1.6	
" heat-precipitated.....	0	1.6	2.6	
" heat-destroyed.....	100	1.3	1.4	
" trypsin.....	100	0.5	1.2	
" "	100	0.6	1.2	
" " acidified.....	100	0.7		
" nitrite.....	15	0.9	1.2	
" "	30	1.1	1.5	
" iodine.....	80	1.1	1.3	
" I, 0.0030 mg. N per unit.....	0	1.6	2.7	
" nitrite.....	0	1.4	1.9	
" "	50	1.4	1.8	

Table X. This insulin was made by our usual method of purification and analyzed 0.0055 mg. of nitrogen per unit. In every case in which the insulin was inactivated, about half the sodium hydroxide labile sulfur was destroyed. The agents which destroyed the potency were benzoyl chloride, alkaline diazo compounds, phenylhydrazine, ultra-violet light, heat, trypsin, and

iodine. In addition, benzoylation, coupling, ultra-violet light, and trypsin affect the carbonate sulfur. When the insulin is destroyed by heating in acid solution, there is no change in the sodium carbonate sulfur. Abel and Geiling made the same observation upon their insulin. They do not report on the sodium hydroxide sulfur. Before we had succeeded in destroying the sodium hydroxide sulfur without affecting the potency, we were inclined to believe that this form of sulfur was involved in the active insulin group. The observation that anything which destroyed the potency also destroyed this form of sulfur appeared very significant. We almost overlooked the fact that in another insulin (see Table VIII, Insulin II) there is only a very small difference between the sodium hydroxide and sodium carbonate sulfurs.

When the insulin was inactivated by exposure to ultra-violet light, there was at first a marked decrease in the sodium hydroxide and sodium carbonate labile sulfurs, followed by a small but definite increase of both these values. At the end of 17 hours, half the potency was destroyed with an absolute 0.5 per cent decrease in sodium carbonate labile sulfur. 15 more hours of exposure destroyed 15 per cent additional potency, and 30 more hours, an added 25 per cent of the potency. The sodium carbonate labile sulfur at the end of this period was raised almost to its initial value. This is evidence for the occurrence of at least two reactions in the insulin complex. The experiment was performed in an atmosphere of nitrogen. An autooxidation-reduction is brought about by the action of the ultra-violet light, the active insulin group being reduced at the expense of the labile sulfur. When the potency has been destroyed, the labile sulfur is reduced at the expense of some other part of the molecule. This is an explanation based upon the facts; but there is no absolute proof for it. Ultra-violet light will attack almost any organic compound, even the most stable of chromophoric groups. What happens is usually a matter of speculation. Ultra-violet light changed neither the pH of the insulin nor the test for cystine.

The experiments relating to the action of trypsin upon the labile sulfur were significant because they showed that the sodium carbonate labile sulfur was already liberated by the trypsin, and that acidification, without the carbonate heating, gave the same

amount of sulfur as heating with carbonate in the usual way. Some sodium carbonate labile sulfur and a large percentage of sodium hydroxide labile sulfur are destroyed by tryptic digestion, possibly through oxidation by the air. Johnson (15) has suggested that the labile sulfur may be present in protein as a thiocarbonyl linkage in thiopolypeptides and dithiopiperazine derivatives. It is interesting to conceive of trypsin attacking these compounds and disrupting them through the elimination of hydrogen sulfide.

Phenylhydrazine is cited by Laqueur (16) as an insulin precipitant. We found that it destroys about 60 per cent of the potency and a large percentage of the sodium hydroxide labile sulfur; it also renders the product acid-insoluble. Attempts at regenerating the insulin by separating the phenylhydrazine through acetone, as accomplished in sugar chemistry, were unsuccessful.

The great importance attached to the results with nitrous acid led us to repeat the experiments upon another sample of insulin. We used Insulin I, purified, which had twice the potency, per nitrogen basis, of the other preparation. The results were just as significant. When small amounts of nitrous acid were employed, none of the potency was destroyed. The sodium hydroxide sulfur was lowered from 2.7 to 1.9 per cent and the carbonate sulfur was practically unchanged. When more nitrous acid was used, 50 per cent of the potency was destroyed but there was no further change in the sulfur values. This is another instance of inactivation of the active group through some group other than the labile sulfur.

Experimental.—The micro method devised in this laboratory for the determination of the labile sulfur is described in a separate paper (17). We have followed Abel's conditions of using 0.1 N sodium carbonate and 10 per cent sodium hydroxide in an atmosphere of nitrogen. In Abel's method, the sulfur is separated by contact with plumbite. Preliminary experiments showed that the separation as lead sulfide was unnecessary. We check Abel's cystine value exactly. At least seven of our insulins, which were of the same degree of purity as Abel's, yielded figures very similar to those obtained by him, the sodium carbonate sulfurs approximating 1 per cent, the sodium hydroxide 2 per cent, and the total sulfur 3 per cent. With the exception of the biuret-free insulin, all of the data pre-

sented have been checked from 2 to 5 times. The preparations of the chemical insulin derivatives used in the labile sulfur analyses have been considered under their respective headings. The keratin azo compound was prepared under the same experimental conditions as the insulin derivative. In all the samples used for sulfur analyses, special care was exercised in separating excess reagent from the sample. The samples treated with nitrous acid were not analyzed for sulfur until they gave a negative test with starch-potassium iodide solution. The benzoylated product was extracted with ether and centrifuged from dilute alkaline solution. It might be argued that the sulfur analyses are lower in the chemically treated insulins, not because the sulfur has been destroyed, but because the sulfur liberated in the alkaline heating reacts with the new chemical derivative. In answering this objection it is necessary to consider each case separately. Since the carbonate sulfur before and after nitrous acid treatment is the same, the possibility of a reduction of a nitroso compound is negatived. The possibility that the sulfide reacts with the azo group is remote, it being a common practice to reduce nitroazo compounds to aminoazo compounds with sulfide. For this reason the use of para-nitrobenzenediazonium chloride and the arsenic analogue of sulfanilic acid, though excellent coupling reagents for insulin, was avoided. The experiments with ultraviolet light were performed in a quartz tube in an atmosphere of nitrogen.

General Considerations.

Our usual preparations of insulin respond to the customary protein reactions and are precipitated by the ordinary protein precipitants. This purified insulin is soluble on either side of the isoelectric point, which lies at a pH of 5.8 to 6.0 (colorimetric). Most of the active material is not dialyzable. Sulfur is present in other forms beside cystine. Phosphorus is absent. Carbohydrate is not present. Pepsin and trypsin inactivate the insulin. Hydrolysis with acids yields a distribution of the amino acids which is characteristic of protein and of insulin, as reported by others.

Certain observations suggest that insulin is not a protein but that the properties just mentioned are chiefly due to contaminating protein material. The nitrogen content of several preparations is low for a typical protein. Values ranging from 12 to 13 per cent are not uncommon. Higher values were found for tyrosine and cystine than others have reported. A sample analyzing 25 units per mg. showed 17.9 per cent tyrosine and 7.1 per cent cystine by the method of Folin and Looney (18). A different preparation, which also contained 25 units per mg.,

gave values of 12.9 per cent tyrosine and 6.6 per cent cystine. These may be compared with Scott's figures of 10.0 per cent tyrosine and 0.7 per cent cystine. The higher values indicate a concentration of these amino acids in the particular insulins examined. In these preparations one-fifth of the material was composed of the two amino acids. Such values are certainly unusual for proteins. Several experiments already presented have shown that very small amounts of different reagents inactivate the insulin. The quantities required to inactivate insulin are much smaller than the amounts necessary to react completely with the total complex. Such experiments indicate that the physiologically active group comprises but a fraction of the ordinary purified preparations of insulin.

Murlin (6, 19) has reported the preparation of biuret-free insulin from pancreas perfusates. The earlier observation was severely criticized by various workers who maintained that not enough material was used to obtain the biuret test. In his second paper, Murlin has met this objection by using such large amounts of material as to leave no doubt about the correctness of the observation. We have prepared biuret-free insulin from perfusates according to Murlin's method and from our purified insulins by application of the same principles. 20 units of ordinary protein insulin having a potency of 50 units per mg. give a positive biuret test. 80 units of our biuret-free insulin do not give the faintest trace of a test under the same conditions. Whether this biuret-free insulin is the partially inactivated hormone, freed from protein, or whether it is merely a denatured protein, has not been established. But the fact remains that the physiologically active group is not dependent for its activity upon the characteristic biuret linkages.

SUMMARY.

A method for the preparation of insulin is described. This method gives a good product with comparatively less effort than is required by other procedures.

Various methods of purifying crude insulin are discussed. Fractional precipitation with alcohol is a very efficient treatment

for eliminating inactive material and for concentrating the hormone.

The action of several chemical reagents upon insulin is considered. The important fact established by these experiments is that the quantities of several different reagents required to inactivate insulin are much smaller than the amounts necessary to react completely with the total complex. The experiments indicate that the physiologically active group comprises but a fraction of the ordinary purified preparations of insulin.

A rather complete study of the labile sulfur of insulin and other proteins was made. The results of these experiments indicate that the apparent relationship between the activity of insulin and the labile sulfur is fortuitous. A large percentage of the sodium hydroxide labile sulfur can be destroyed without affecting the physiological activity. Furthermore, the potency of insulin can be destroyed without changing the sodium carbonate labile sulfur.

The hormone, insulin, probably comprises but a fraction of all purified preparations which contain protein.

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