

<sup>3</sup> Jahn, *Zs. anorg. Chem.*, **60**, 337 (1908). Kailan and John, *Zs. anorg. Chem.*, **68**, 243 (1910).

<sup>4</sup> It must be pointed out that this is a consequence only if we also reject chain reactions in which consecutively normal ozone molecules are decomposed for the activation of some molecule in successive steps, which, after a sufficient number of such steps, may clearly be made to possess enough energy to account for the observed excitation energies. Chain mechanisms of this type, and of such a design as to actually account for the observed radiation appear, from energy considerations and our knowledge of the kinetics of ozone decomposition, to be highly improbable.

<sup>5</sup> By this activation there is understood any passage of the ozone molecule to a form of higher energy. Thus a possible form of activation would be  $O_3 \longrightarrow O_2 + O$ .

<sup>6</sup> Trautz and Seidel, *Ann. Physik*, **67**, 527 (1922).

<sup>7</sup> In regard to the possibility of reaction between ozone and impurity affording sufficient energy to account for the luminescence observed without any activation of these reactants, it may be pointed out that if it be assumed, in view of the work of Trautz and Seidel referred to above, that it is the reaction between ozone and carbon monoxide which yields the energy that excites the luminescence, the conclusions of this paper remain substantially unchanged. For, while this reaction affords more energy than the reaction of two normal ozone molecules, it does not give a sufficient amount to account for the short wave-length radiation that is observed. Thus from thermochemical data it may readily be computed that the reaction  $O_3 + CO = CO_2 + O_2$  is attended by an energy liberation of 102,500 calories. This might account for radiation of wavelengths as short as 2780 Å, but not shorter. As has been said above, Stuchtey finds an important part of the total radiation to be of considerably shorter wave-length than this, namely that lying in the vicinity of 2540 Å, and radiation extending even farther in the direction of shorter wave-length than this latter is also observed. Therefore the conclusions of this paper can be applied equally as well to the reaction between ozone and carbon monoxide, if this reaction be thought responsible for the luminescence.

## CRYSTALLINE INSULIN\*

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In two preceding communications<sup>1,2</sup> it has been told how my collaborators and I were induced to attack the problem of the isolation of insulin and what progress had been made towards its solution to within a few months past. It was there pointed out that a clue had been found, the following out of which promised to lead to the desired goal. This clue eventuated from the observation that when the highly impure and complex, though therapeutically serviceable insulin extracts of commerce are boiled for a short time with *N*/10 sodium carbonate the resulting physiological inactivation of the extracts is always associated with an alteration of a part of their sulphur, an element which our experiments justified us in believing to be an integral constituent of the hormone itself. After

such treatment with a weak alkali a new property appears in the altered insulin in that it now shows an extraordinary sensitivity to very dilute acids which immediately, and contrary to their usual action, liberate hydrogen sulphide from it. It was found that inert fractions prepared from such extracts of the pancreas contain very little of this labile sulphur and a table was constructed which showed that the labile sulphur of a preparation is directly proportional to its hypoglycaemic activity for experimental animals. In other words, the higher the amount of labile sulphur in a given preparation the greater its potency in lowering the percentage of blood sugar.

The methods usually employed in fractioning and purifying biological mixtures have been found to be quite useless in the attempt to separate the hormone from the numerous impurities that are associated with it in even the best of the extracts employed in medical practice. Equally unsuitable to the purpose in mind was the employment of the method of isoelectric precipitation from pH 4.7 to 5.0 no matter how frequently repeated, as likewise the analogous method of electro-dialysis—a method which has not yielded results of any particular value in the hands of those who have tried it out on insulin extracts. We are here dealing with an amphotolyte present in very small amount in a solution of other amphotytes whose solubility and precipitation coefficients lie very close to one another. It seemed worthwhile to the writer therefore to employ only weak bases and acids, more especially such as can act as buffers, in the hope that there might result from their use such an alteration of solubilities, that impurities would either fall out of or remain in solution while the hormone in each case took the opposite course. The successful outcome of the experiments proved the essential correctness of the idea.

For a description of our earlier methods of purification the reader is referred to the first paper of this series. The employment of the simple method there described, led uniformly to the preparation of an insulin fraction, Fraction IV, with a rabbit unitage of 40 or more units to the milligram, a unitage that has lately been increased by a more skilful use of these earlier methods. The next steps in the process leading directly to the crystallization of insulin are as follows.

One gram, approximately, of the so-called Fraction IV, is dissolved in a little more than the required volume of *N*/6 acetic acid, enough water is added to bring the volume up to 60 cc. or thereabout, and the contaminating substances (together with some insulin) are then precipitated by the addition of an acidulated solution of brucine containing 6 gms. of brucine in 95 cc. of *N*/6 acetic acid. The resulting clear supernatant fluid which contains nearly pure insulin is separated from the precipitate by centrifugalization. Insulin remaining in the precipitate may be removed by dissolving in *N*/6 acetic acid and precipitating with the brucine solution

as before. How profitable it may be to repeat the process has not been determined at the moment. The clear colorless centrifugalate containing the insulin is then precipitated with *N*/6 pyridine and the precipitate and fluid are immediately centrifuged. The precipitate which settles out is largely crystalline in character, the sides of the tube are found to be coated with glistening highly refractive crystals and the topmost layer of the precipitate consists of similar crystals. It is not a difficult matter to remove this topmost layer of crystals by means of a rubber-mounted

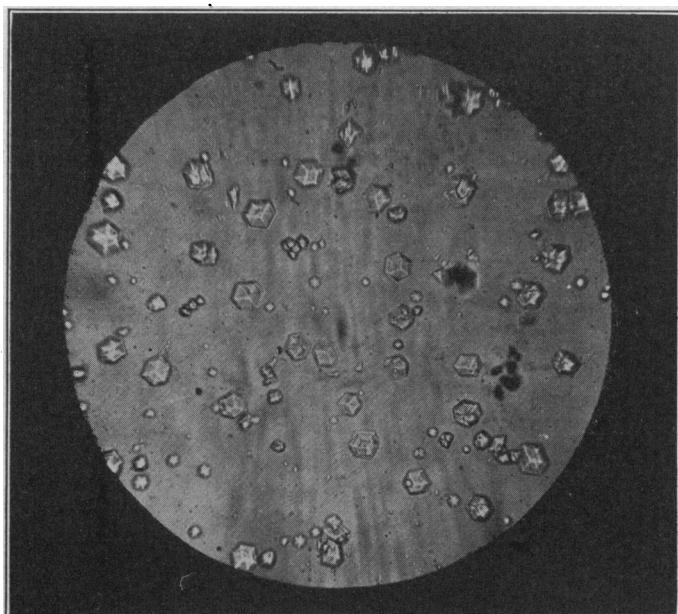


FIGURE 1  
Crystals precipitated from acetic acid solution with *N*/6 pyridine.

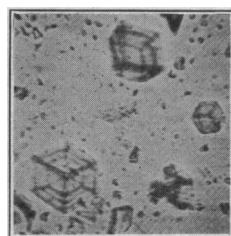


FIGURE 2  
Crystals precipitated  
from  $\text{Na}_2\text{HPO}_4$  solution  
with *N*/6 acetic acid.

Figures 1 and 2. Photo-micrographs, magnification 460  $\times$ , taken by transmitted light.

pipette and to free them from adherent pyridine and acetic acid by frequently washing them with distilled water at room temperature in which medium pure crystalline insulin is quite insoluble. The crystals which are of the type shown in figure 1 may be recrystallized by dissolving them in *N*/6 acetic acid and reprecipitating with *N*/6 pyridine. It has been my practice, however, of late to dissolve this crystalline insulin together with whatever fine granular material there happens to be mixed with it in *M*/15 disodium hydrogen phosphate. To the clear solution *N*/6 acetic acid is added drop by drop with vigorous shaking in order that any insulin thrown out will at once pass into solution again, and this addition of the acid is continued until a slight amount of turbidity persists per-

manently. The flask is then carefully set aside overnight. On the following morning the bottom of the flask as well as its sides are coated with an abundance of colorless, highly refractive crystals. The small amount of amorphous insulin that falls out in very minute granules or floccules—an expression of the above-mentioned turbidity—is readily separated by means of the pipette during the washing with cold water, as the compact crystals obtained by this method sink quickly to the bottom of the tube like so much sea sand. This process of crystallization may be repeated as often as is thought to be necessary. Only a few experiments have as yet been made with the object of crystallizing the compound from hot solvents, and to prepare crystalline derivatives.

Professor Swartz of the Department of Geology of the Johns Hopkins University has kindly examined samples of the insulin crystals shown in figures 1 and 2 and reports them as being very uniform in character suggesting a high degree of purity, as doubly refracting and as belonging to the rhombohedral division of the hexagonal system. The crystals which are allowed to form more slowly out of acidulated disodium hydrogen phosphate solution are very much larger than those precipitated quickly by pyridine out of an acetic acid solution, such crystals being readily perceptible to the naked eye as individual crystals. Irrespective of the manner of their preparation the crystals melt sharply at 233°C. with slight browning occurring at 215°C. The melting point has remained constant on recrystallization.

*Chemical Reactions.*—A large fraction of the sulphur of the molecule is in the labile state previously described as characteristic of all insulin fractions of high rabbit unitage. Quantitative analyses of both the labile and more firmly bound sulphur are in progress. The compound gives a beautiful biuret reaction passing from a clear pink to a fine purple on further addition of copper sulphate to the alkaline solution. Millon's reaction is unequivocal and positive. The Pauly and the ninhydrin reactions are also positive. This pure insulin is very sensitive to alkali, as shown by the fact that boiling for 15 minutes with *N*/10 sodium carbonate deprives it entirely of its hypoglycaemic activity. It will be seen that the chemical reactions here described are of considerable significance as shedding light on the chemical structure of the compound. It is now being examined in respect to its other physical and chemical properties and efforts will also be made to identify its various cleavage products.

*Animal Experiments.*—Numerous tests have already been made in order to establish the exact rabbit unitage of the crystalline compound. The rabbit unit employed by us is that fraction of a milligram per kilogram of body weight which will cause convulsions or lower the blood sugar to 0.045 per cent within 5 hours. This corresponds to approximately 1 $\frac{1}{2}$  times the "clinical unit" used at present.

The exact dosage of the crystalline material has not yet been established, but for the present, as is shown in the subjoined table (1), amounts as small as  $\frac{1}{100}$  of a milligram per kilo lowered the blood sugar to about the convulsive level, 0.045 per cent. In another series of experiments  $\frac{1}{125}$  of a milligram per kilo produced convulsions in one rabbit and lowered the

TABLE 1

## BLOOD SUGAR DETERMINATIONS

Insulin crystals dissolved in  $M/15 \text{ Na}_2\text{HPO}_4$ .

1 mgm. equivalent to 10 cc. solution.

Injections were made subcutaneously.

NO.	WT. KILOS	DOSE PER KILO MG.M.	ACTUAL DOSE CC.	TIME	BLOOD SUGAR—MG.M. PER 100 CC.					REMARKS
					INITIAL	1 $\frac{1}{2}$ HRS.	3 HRS.	5 HRS.	UNITS <sup>a</sup>	
44	2.07	0.016	0.345	11.23	Lost	50	70	88	60	
39	1.75	0.0125	0.22	11.29	113	32	54	79	80	
42	1.62	0.0125	0.2	11.35	97	50	54	102	80	
15	1.54	0.011	0.17	11.38	113	34	39	47	90	
29	1.51	0.01	0.151	11.43	110	57	92	110	100	
12	1.93	0.01	0.193	11.48	125	48	57	79	100	

TABLE 2

14	1.6	0.008	0.13	11.06	119	25	..	..	125	Convulsion
46	1.95	0.008	0.15	11.11	115	56	77	111	125	

<sup>a</sup> As defined in the text.

blood sugar almost to the convulsive level in a second animal. (Table 2.) It will be clear, therefore, that we are dealing here with a hormone which, in very high dilution, influences carbohydrate metabolism acting as it were like a catalyst in a chemical reaction.

With the isolation of this hormone in crystalline form we are leaving the region of qualitative experiments, and may look forward to quantitative results both in the biological and chemical field which the future promises to yield.

My collaborators in this field of work, Dr. E. M. K. Geiling, Dr. C. A. Rouiller and Dr. David Campbell and I hope to be able in due season to publish more extensive reports on the various researches which naturally arise in the investigation of the properties, chemical and biological, of the pure product.

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<sup>1</sup> Abel, J. J., and Geiling, E. M. K., *Jour. Pharm. and Exper. Therap.*, **25**, 423, 1925.

<sup>2</sup> Abel, J. J., Geiling, E. M. K., Alles, G., and Raymond, A., *Science*, **67**, 169, 1925.