In a comparison of the amount of vit. B_{12} in different tissues, the kidney contained more than the liver with the exception of the group receiving liver "L," which is in agreement with the work of Lewis, Register, and Elvehjem(1). When the diet contained liver "L" the liver contained slightly more than the kidney. The spleens contained approximately the same amount as the kidney with the exception of those in the basal groups. The spleens of this group contained slightly more than half that in the kidney. The amounts in the heart were relatively small compared with that in the other tissues.

The amounts of vit. B_{12} in different samples of tissue from the same litter are given in Table III. There it may be seen that the values were in the same range, with the exception of those for the spleen in litter 1, for all the samples within a litter.

Summary. The amount of vit. B_{12} in the liver, spleen and heart of 28-day-old rats was approximately doubled when a source of vit. B_{12} was added to the diet of the mothers. The diet of the mother had no important effect on the vit. B_{12} content of the kidney. In general the kidney and spleen contained slightly more vit. B_{12} than liver. The heart contained a relatively small amount of vit. B_{12} in comparison to other tissues.

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Infra Red Spectroscopy of Tissues. Effect of Insulin Shock.* (18458)

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Infra spectra of organic compounds are with a few exceptions, highly characteristic and specific. For this reason study of infra red spectra of tissues has been considered as an adjunct method of tissue analysis(1). In our attempt at evaluating the potential use of infra red spectroscopy for tissue studies 2 characteristics of this physical method became apparent. 1. The locations of absorption bands of molecules are so extremely sensitive even to slight changes of molecular structure that it is necessary to resort to empirism and to draw conclusions about the structures by correlation between bands which occur regularly with recurring structural features throughout a series of material examined. 2. The infra red energy emitted by all commercial instruments is so small that loss of energy as may occur in examination of very small structures may make qualitative observations inaccurate and quantitative studies futile. The present paper is a report on our method for infra red spectroscopy of tissues, on qualitative spectroscopy of various tissues especially neural tissue, and on quantitative infra red spectroscopy in brain tissue of control rabbits and rabbits in insulin shock. For this study we have selected to investigate tissue masses rather than smaller units. The selection of brain areas, *viz*: diencephalon, hippocampus, cerebellum, and tectum was largely governed by the clinical manifestations of disordered neural functions displayed during insulin shock.

Preparation of tissue sections. The organs of the animals were rapidly frozen with dry ice immediately after the animal was killed, in almost all instances, by injection of air into the heart. For spectroscopy, frozen sections of 50 μ and, for some purposes, 25 μ thickness prepared by the technic of Adam-

* This work was aided by a research grant to the Diabetic Coma Project from the National Institute of Health, U. S. Public Health Service.

1. Blout, E. R., and Mellors, R. C., *Science,* 1949, v110, 137.

stone and Taylor(2) were placed on silver chloride plates and dried at low temperature. Strict adherence to this technic is necessary, as is the selection of homogenous sections. As a check on the exact location of areas studied spectroscopically and to rule out the possibility of complicating disease, histologic preparations were made from the block remaining after cutting the tissue for spectroscopy.

Method of qualitative infra red spectroscopy. For recording of the spectra with the Beckman IR2, infra red spectrophotometer, the silver chloride plate was placed in a cell holder, the desired part of the section centered, and then covered with a specially designed mask so that no infra red energy would pass through any area of the tissue or part of the plate not covered by the section. Most of our tissue sections were obtained from an area of 8 x 5 mm. In some instances, spectra were recorded from larger, not infrequently, from smaller areas. Areas as small as 3 x 3 mm. can be studied without exceeding the proper signal to noise ratio of the instrument. Blanks were recorded from every silver chloride plate using masks with the same surface area and the same slit width of the instrument. Proper corrections were applied for the different amplifications.

Results of qualitative infra red spectroscopy of the tissues of rabbits. The results given here represent a selection of data obtained in a study of spectra from well over 500 tissue sections of control rabbits and rabbits in insulin shock. A large number of spectra were recorded from different parts of the brain (cerebellum, hippocampus, tectum, diencephalon, white matter, grey matter), a smaller number from liver, heart, medulla and cortex of the kidney, adrenal, thymus, and spleen, and in a few instances from optic nerve and lymph node. The recordings from 15-2.8 μ showed a region below 8.06 μ (1241 cm^{-1}) with several more or less strong bands which can be found in almost all tissues. These bands, with the exception of the band at 8.06 μ for which no definite assignment has yet been made, may be correlated with the known "protein polyamide" vibrations, *viz;* 3.04 μ (3209 cm^{-1}) N H stretching; 3.4 μ (2940 cm^{-1}) C H bending; 6 μ (1670 cm^{-1}) C O stretching, and the deformation vibration of N H at 6.44 μ (1553 cm^{-1}) and the deformation vibration of C H at 6.88 μ (1450 cm^{-1})(3). In the region of higher wave length (lower frequencies), the tissue spectra showed a fingerprint region which is so specific for individual organs that it was frequently possible to identify the tissue from the infra red spectra. Table I shows the location of bands from 11-8 μ in control rabbits and rabbits in insulin shock.

Several hundred sections of neural tissues examined by infra red spectroscopy showed without exception the same spectral pattern, *e.g.,* a weak band at 10.27-.30 μ and a strong band at 9.31-.35 μ. It is noteworthy that these bands could be seen at exactly the same locations in sections from different parts of the brain, sciatic nerve, and optic nerve of the rabbit, in the brain tissue sections from fresh human post mortem material, in the brain sections obtained from an anesthetized dog and that the strong band at 9.31-.35 μ was previously found by other investigators in the nerve of the frog(4). Great differences, however, existed in the strength of these bands. This will be discussed with quantitative determination of infra red spectra.

A relative small number, *e.g.,* a total of 76 visceral tissue sections were studied and only noteworthy findings can be discussed. More details are contained in Table I. A band which was very similar in strength to the weak band found in neural tissues was observed at somewhat similar location (10.32 $\pm$ .06 μ) in all visceral tissues except the liver of control animals. The visceral tissue spectra showed also one or more additional bands between 9.89 and 9.16 μ. The locations of these bands were distinctly different from those found in neural tissue (9.31-.35); they were somewhat similar in sections from thy-

2. Adamstone, F. B., and Taylor, A. B., *Stain Technology,* 1948, v23, 109.

3. Darmon, L. E., and Sutherland, G. B. B. M. Y., *Am. Chem. Soc.,* 1947, v69, 207.

4. Barer, R., Cole, A. R. H., and Thompson, H. W., *Nature,* 1949, v163, 198.

TABLE I. Location of Bands from 8-11 μ in Animal Tissue.

Type tissue	No. rabbits	Location and strength† of bands					
Brain, optic sciatic N.*	25	10.30-.272		9.35-.314			8.063
Liver							
Control	5	10.271	9.70-.644		9.23-.212	8.64-.632	8.073
Insulin shock	5	10.30-.282			9.23-.214	8.64-.611	8.063
Insulin no shock	2	10.35-.282			9.23-.224	8.602	8.05-.033
Insulin no shock	3	10.261	9.66-.624		9.22-.212	8.66-.632	8.08-.063
Insulin rapid shock	2	10.27-.261	9.68-.664		9.22-.212	8.642	8.063
Heart							
Control	3	10.30-.271		9.504	9.24-.222	8.561	8.07-.043
Insulin shock	4	10.27-.261		9.54-.524	9.25-.182	8.61-.561	8.08-.053
Insulin shock	1	10.281		9.501	9.204	8.561	8.063
Kidney-Medulla							
Control 4							
Insulin shock 3	7	10.311		9.47-.444			8.043
Insulin shock	6	10.33-.271			9.234		8.073
Kidney-Cortex							
Control 3							
Insulin shock 5	8	10.31-.281-2			9.30-.224 (Broad)		8.073
Adrenal							
Control	3	10.35-.301	9.822	9.60-.562	9.26-.173	8.52-.491	8.053
Insulin shock	7	10.38-.261	9.89-.842	9.651	9.27-.233	8.491	8.053
Repeated insulin shock	1	10.381	9.892	9.651	9.381	8.511	8.053
Thymus							
Control 3							
Insulin shock 5	8	10.36-.332			9.22-.164		8.063
Spleen							
Control 3							
Insulin shock 7	10	10.38-.302			9.22-.234		8.073
Lymph node							
Insulin shock	1	10.382			9.174		8.073

* Control and treated animals gave same results. † = 1 very weak, 2 weak, 3 medium, 4 strong.

mus, spleen and lymph node (9.23-.16), but clearly distinguishable in all the other tissues. A strong band at 9.64-.70 μ and a weak band at 9.21-.23 μ, for example was only seen in liver tissue. Some visceral tissue sections from animals which had received insulin showed a shift of the strong band toward lower wave length. This was especially apparent in the liver from 7 insulin animals in which the band at 9.64-.70 μ failed to appear and the band at 9.21-.23 had become very strong, the strongest band of the finger print region. Larger number of animals of different species must be studied in order to verify these findings.

Structural assignments of the tissue spectra. Blout suggested similarity of infra red spectra of nucleic acids and certain bands he found in a few tissue spectra. We have studied infra red spectra of ribose nucleic acid, desoxyribose nucleic acid, d-ribose, d-desoxyribose, nucleotides, purine- and pyrimidine derivatives and compared our data with those of Blout(5), Clark(6), and Randall *et al.*(7) and will report our findings in detail elsewhere. We feel that although about 100 different compounds related to nucleic acid metabolisms have been evaluated still more pure compounds and mixtures of pure compounds will have to be studied before any definite assignments of tissue spectra should be attempted. For the tentative assignment of the weak band found in neural tissue at 10.27-.30 μ and in visceral tissues at 10.32 $\pm$ .06 μ, the

5. Blout, E. R., and Fields, M. J., *Biol. Chem.*, 1949, v178, 335.

6. Clark, C. C., Infra red absorption and x-ray diffraction of nucleic acid derivatives, University Columbia 1950 P.H.D. Publication 1838.

7. Randall, H. M., *et al.*, Infra Red Determinations of Organic Structures, D. Van Nostrand, New York, 1949.

following findings in pure compounds may be significant: A strong band was found at 10.35 μ in ribonucleic acid, at 10.28-.30 μ in desoxyribonucleic acid (Bios), and at 10.25 μ in adenosine, weaker bands were found at 10.40 μ in xanthine, at 10.37 μ in hypoxanthine, at 10.26 μ in adenine sulfate, and at 10.25 μ in A. T. P. (di barium salt). Uric acid did not show any band at all at this wave length range. In the other part of the finger print region (9.89-9.16 μ) where most of the tissue spectra showed rather strong bands, differences between d-ribose and d-desoxyribose (Bios) were apparent. D-ribose showed a weak band at 9.19-.23 μ and a strong band at 9.63 μ; d-desoxyribose on the other hand showed a strong band at 9.22 μ and a weak band at 9.82-.85 μ. The spectra of liver tissue from control animals were very similar to those of d-ribose. When a shift of the strong band appeared in liver tissue from animals which received insulin that band had shifted to 9.22-.20 μ; that is almost exactly the location of the strongest band of d-desoxyribose. Evaluation of the strong bands of thymine, uracil, guanine hydrochloride, adenine sulfate, xanthine, hypoxanthine, and uric acid proved that none of those compounds possessed any band comparable with strong bands of the tissue spectra between 9.70 and 9.16 μ. Adenine sulfate showed a medium strong band at 9.78 μ and uric acid a medium strong band at 9.75 μ. It is, therefore, improbable that any of the strong bands found in the tissues at this wave length range, may have been caused by free purines or pyrimidines. Nucleosides, nucleotides, and nucleic acids, however, showed a large number of bands which may have to be considered for the assignment of the strong bands in the tissue spectra. The locations of strong bands of some of the pure compound are as follows: adenosine 9.63 μ, uridine 9.46 μ, cytosine sulfate 9.28 μ, yeast adenylic acid 9.75 μ, yeast guanylic acid 9.50 μ, uridylic acid 9.71 and 9.57 μ, cytidilic acid 9.25-.26 μ, ribonucleic acid 9.56 μ, and desoxyribonucleic acid 9.89 and 9.39 μ. In an attempt at obtaining further information about the structures which produce the infra red spectra in neural tissue

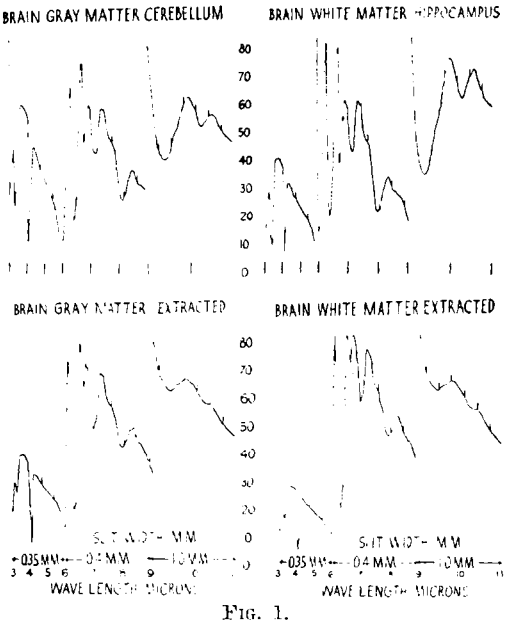

FIG. 1.

extraction experiments similar to those reported by Barer, Cole and Thompson were carried out. Fig. 1 shows the spectra of white and grey matter of the brain before and after extraction with a mixture of ether and chloroform. It is clearly visible from this figure that the bands at 10.27-.30 μ and 9.31-.35 μ, which were so characteristic for the neural tissue, completely disappeared after the extraction, that the unassigned band at 8.06 μ was greatly diminished, and that polyamide vibrations as those at 6.44 μ (deformation vibration of NH) were not influenced by the extraction at all. Barer *et al.*, have already pointed out that their observations (frog nerve tissue) do not necessarily mean that the substances which had been removed by the extraction had to be lipids. They suggested that the reduction of the band at about 9.30 μ was also consistent with the removal of -c-o- bonds such as occur in esters and implied that the band near 8.10 μ which they also found greatly reduced by the extraction appeared to be rather in the polyamides than the lipids.

Quantitative infra red spectroscopy: the effect of insulin on the infra red spectra of brain tissue. The great constancy and reproducibility of infra red spectra of neural

tissues from healthy animals justified an attempt at quantitative estimation of those structures which produce the characteristic spectra of brain tissue of control animals and at comparison of data thus obtained with results of quantitations of brain tissue spectra from animals in insulin shock. The fact that the substances producing the characteristic bands in neural tissue, *viz:* at 10.27-.30 μ and at 9.31-.35 μ could be removed by chloroform ether extraction while the band at 6.44 μ (deformation vibration of N H) was not influenced by the extraction at all, indicated that this band of the polyamide vibrations (6.44 μ) could be used as an internal standard for the quantitative estimation of the substances causing the vibrations at 10.27-.30 μ and at 9.31-.35 μ. It was assumed that the ratio of optical densities $\frac{D\ 10.30}{D\ 6.44}$ called K_1 and $\frac{D\ 9.35}{D\ 6.44}$ referred to as K_2 were independent of the thickness of the sections and the "protein back ground," and that they were true relations of the concentration and the molecular extinction of the structures producing the vibrations at 10.27-.30 and 9.31-.35 μ. The correctness of these assumptions was proved by the fact that sections of the same tissue but of different thickness (25 and 50 μ) and duplicates of the same tissue gave almost identical K_1 and/or K_2 figures. It should be pointed out, however, that measurements and calculations agree with these assumptions only if they are carried out with great accuracy, in bands which are clearly distinguishable. Whenever sharply defined bands are not visible, indicating very small concentrations of these structures, these calculations may give erroneous conclusions, and might better be replaced by well worked out base-line method(8).

In a larger number of sections through different parts of the brain, the K_1 values varied between .21 and .45 and the K_2 values between .47 and .78. This shows greater variation than would actually exist if the same

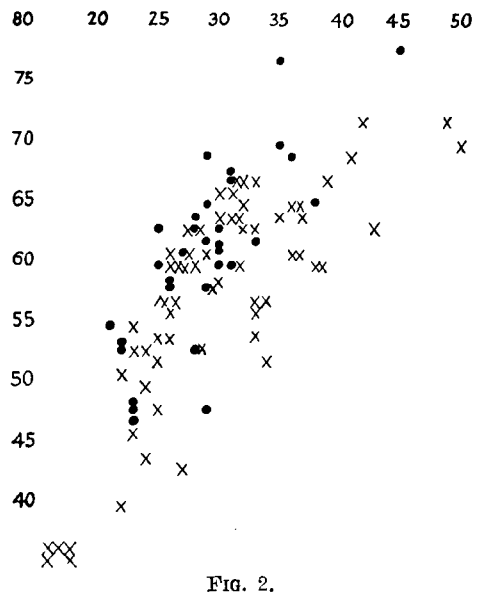

FIG. 2.

parts of the brain, *e.g.* hippocampus, tectum, cerebellum, and diencephalon were compared. Especially large spectral differences between grey and white matter of the brain were apparent. In one experiment, for example, the grey matter section showed a K_1 of .25 and a K_2 of .57; the white matter gave a K_1 of .31 and a K_2 of .78.

The K_1 and K_2 values of brain tissues (hippocampus, tectum, cerebellum, and diencephalon) from 7 control rabbits and 10 rabbits in insulin shock are shown in a distribution graph in which K_1 (abscissa) is plotted against K_2 (ordinate) (Fig. 2). The full dots indicate the figures obtained from the brain tissue of controls and the crosses, those from tissue of animals in insulin shock. In the majority of the experiments (6 out of 10) some of the series of brain sections from animals in insulin shock showed significantly different K values when compared with the K values from normal rabbits. In 5 brain sections of rabbits in insulin shock, the K_1 and/or K_2 figures were either so low that accurate calculation was impossible or the K values were actually zero. Comparison of the actual spectra of the same brain area of a control and a rabbit in insulin shock (Fig. 3) clearly shows these fundamental differ-

8. Wright, N., *Ind. Eng. Chem.*, 1941, v13, 1.

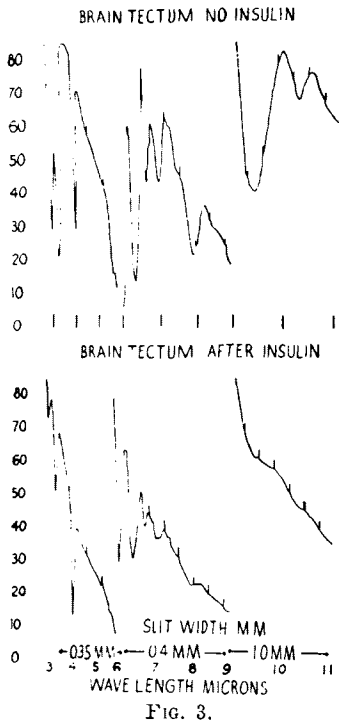

FIG. 3.

ences. In 4 other brain sections of rabbits in insulin shock, the K_1 and K_2 figures were considerably lower than those found in any brain section of control animals. A shift in the relation of K_1 to K_2 occurring in other brain sections from animals in insulin shock is also indicated in the graph and needs further evaluation. In 3 animals free of shock symptoms after insulin injection, sections through different parts of the brain gave about the same K figures as found in control rabbits.

Summary. 1. A method for recording of infra red spectra from frozen tissue section of 25-50 μ thickness and from areas as small as 3 x 3 mm is described. 2. The infra red spectra of tissues show a fingerprint region which is so characteristic of the individual organ that it was frequently possible to identify the tissue from its infra red spectrum. 3. The assignment of tissue spectra to chemical structures as nucleic acid derivatives has been discussed. 4. A method for quantitative infra red spectroscopy in brain tissue has been described. 5. Significant spectroscopic changes in brain tissue of animals in insulin shock have been demonstrated.

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Effect of Proteolytic Enzymes and Fixation on Metachromasia of Skin Collagen. (18459)

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In the course of some histochemical observations on generalized and circumscribed myxedema we(1) have had occasion to treat sections of fixed tissue with certain proteolytic enzymes. We were surprised to find that incubation of sections with pepsin and trypsin led to the appearance of metachromatic staining quality in collagen which ordinarily does not display this property(2). This observation has led us to pursue the subject further in order to compare the effects of these two enzymes on unfixed and fixed preparations.

Methods. Human skin, obtained at autopsy or operation, has been used throughout. Frozen sections were made of unfixed material. Some were treated with crystalline pepsin* or trypsin* in the unfixed state; others were fixed in either neutral formalin or 80% ethanol, after which they were washed and treated with the same enzymes. The enzymes were used in a concentration of 5%; the pepsin was dissolved in acetate buffer, pH 5.0, the trypsin in veronal buffer at pH 7.4.

1. Follis, R. H., Jr., To be published.

2. Wislocki, G. B., Bunting, H., and Dempsey, E. W., *Am. J. Anat.*, 1947, v81, 1.

* Worthington Biochemical Laboratory, Freehold, N. J.