rate of fall of activity in heart and in diaphragm is similar in fed rats and in rats that have been starved for 16 hr. Similar rates of loss of activity have also been observed in lung tissue from fed and starved rats. In adipose tissue, the enzyme activity after starvation for 16 hr. has already fallen to about 10% of that in the fed animal (Wing & Robinson, 1967) and in such animals it does not decline further after cycloheximide injection.

It is evident from Fig. 1 that tissue enzyme activities frequently varied considerably from one experiment to another, despite the fact that each value was derived from an assay of the grouped tissues of at least four animals. The reason for this variation is not understood.

The finding that the clearing-factor lipase activity of heart, diaphragm and lung from fed and starved rats and of adipose tissue from fed animals is lost so quickly after cycloheximide injection is most readily explained on the assumption that in such tissues most of the enzyme, or an essential cofactor

or activator thereof, is normally being synthesized and degraded at a rapid rate. However, the possibility that cycloheximide may cause the conversion of the enzyme into an inactive form, without enzyme degradation, cannot be entirely excluded (Wing & Robinson, 1967).

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Quantitative Determination of Free and Conjugated Bilirubin by Diazo Coupling and a Liquid-Extraction and Column-Chromatographic Technique

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Clarification of the relationship between direct-reacting and indirect-reacting bilirubin fractions of the van den Bergh test as constituting bilirubin and bilirubin glucuronide (Billing, 1954; Schmid, 1956, 1957; Talafant, 1956; Billing, Cole & Lathe, 1957) has led to the advent of several techniques for the partitioning of the bilirubin pigments. The reversed-phase partition column-chromatographic technique originally described by Cole & Lathe (1953) utilizing silicone-treated kieselguhr (Howard & Martin, 1950) appeared to be one of the most promising ways to separate bilirubin and its glucuronide conjugate. Although the extraction method used by Schmid (1956, 1957) served its purpose well, it was not quantitative (R. Schmid, personal communication). The purpose of the work now reported was to modify these latter methods to accomplish the dual purpose of a quantitative extraction and a chromatographic separation of bilirubin and its glu-

curonide on a routine basis. In addition, a commercially available column support has now obviated the laborious task of silicone treatment of kieselguhr used as the original chromatography support.

A 3ml. sample of plasma or of tissue homogenate is transferred to a glass-stoppered centrifuge tube and 4.5ml. of 95% (v/v) ethanol and 3ml. of a concentrated diazo reagent (Weber & Schalm, 1962) are added. The stoppered tube is then shaken moderately for 1 hr. Then 9ml. of water, 9.0ml. of butan-1-ol and 1.25g. of crystalline solid Na_2SO_4 are added. The stoppered tube is then shaken vigorously for 15 min. After the shaking, the tubes are centrifuged at 1300g for 5 min. The butanol layer is transferred to a round-bottomed evaporation flask. The layer remaining in the tube plus the protein precipitate is then repeatedly extracted with 2ml. portions of fresh butanol until azo-pigment is no longer extracted into the butanol. The contents

of the evaporating flask are evaporated to dryness with a flash evaporator maintaining the water bath at 30°.

The dry residue is dissolved in 2ml. of butan-1-ol plus 2ml. of distilled water and transferred to a smaller glass-stoppered centrifuge tube. Shaking and centrifugation as previously described are again utilized. This extraction is also continued with 1ml. portions of butan-1-ol until the butanol no longer extracts azo-pigment. The butanol layers are transferred to an evaporation flask and evaporated to dryness as previously described. Extraction and drying are continued until a residue essentially free of salts is obtained on the side of the evaporating flask. This material is then dissolved in polar solvent and applied to the chromatography column.

Polar and non-polar chromatographic solvents are prepared by vigorous shaking of 900ml. of water, 1000ml. of butan-1-ol and 100ml. of 0.1 M-sodium citrate buffer, pH 4.0 (Billing, 1954). Chromatographic columns (300mm. long $\times$ 19mm. inside diam.) are used with sintered-glass disks to support the column material; 40g. of Gas-Chrom CLZ gas-chromatography support (120–140 mesh; Applied Science Laboratories Inc., State College, Pa., U.S.A.) is used for each column. Gas-Chrom CLZ not previously used should be soaked in 95% (v/v) ethanol for 24 hr. and then dried for 24 hr. at 110° before use. The 40g. of Gas-Chrom CLZ is placed in a closed container and 20ml. of non-polar solvent is added. This mixture is vigorously shaken for 5 min. to obtain a homogeneously saturated material. Enough polar solvent is then added to obtain a pourable slurry and the column is packed with pressure to a height of 30 cm. or less with a stainless-steel rod (Martin, 1949). Solvent is maintained above the support material at all times to avoid the occurrence of air bubbles. After application of azo-pigment, the azo-pigment from direct-reacting bile pigment is eluted with polar solvent. Non-polar solvent is used to elute the azo-pigment from indirect-reacting bile pigment.

The Gas-Chrom CLZ column support may be reclaimed and used again after ethanol washing and drying. Satisfactory washing can be obtained by using sequential additions of 95% ethanol to the Gas-Chrom CLZ in a Buchner funnel with slight suction. The last washing is followed by continuous suction for 10–20 min. After oven-drying as described above, the material is stored in a desiccator.

The range of quantities of diazotized pigment

successfully applied simultaneously to a given chromatography column have been from 0.10mg. to 1.25mg. of each component (azo-pigment from both direct-reacting and indirect-reacting bile pigment) and the volume of applied polar solvent containing azo-pigment was from 1 to 5ml. The flow rate for maximum resolution was from 1 to 1.5ml./min. Separation of the two azo-pigments by 10–15cm. is attained on the columns. The mean recoveries obtained in ten experiments with known amounts of added azo-pigment were 99.40% with a coefficient of variation of 3.1% for the azo-pigment from the conjugated component and 99.38% for the azo-pigment from the free component with a coefficient of variation of 8.2%. Extraction without chromatography with known added amounts of ¹⁴C-labelled bilirubin without tissue being present yielded a mean recovery of 98.5% with a coefficient of variation of 2.4%. In early experiments, extraction recoveries from microsomes of a 10% (w/v) liver homogenate was 86.9% with a coefficient of variation of 0.9%.

The extraction and chromatographic methods described in the present communication seem to offer both speed and better quantitative recovery when compared with former extraction and chromatography procedures, with the additional advantage of a commercially available chromatographic support. Total time of elution of both components from the column ranges from 3 to 5 hr. This is much faster than that described by other authors utilizing silicone-treated kieselguhr as a support. When used in this Laboratory, 12–15 hr. was required to elute pigment completely from silicone-treated kieselguhr columns.

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